Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.
Discovering links between elongation factors and general amino acid control in

*Saccharomyces cerevisiae*

*Jyothsna Visweswaraiah*

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

2011
Continuous protein synthesis is essential for life; hence, a steady supply of amino acids must be maintained. In order to respond appropriately to amino acid shortages, cells need to constantly monitor their availability. Cells have a signal transduction pathway, called the general amino acid control (GAAC), for sensing and ameliorating amino acid shortages. Since the sensing occurs on translating ribosomes, the objective of this study was to investigate links between translation elongation and the general amino acid control in *S. cerevisiae*. In all eukaryotes, Gcn2 and its effector Gcn1 are responsible for monitoring amino acid availability. Active protein synthesis requires eukaryotic translation elongation factors (eEFs) to associate with translating ribosomes. This study focussed on two eEFs, eEF3 and eEF1A, and their potential role in GAAC.

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

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

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

(Faith & Perseverance)

- Sai Baba
Acknowledgements

No woman is an island – adapted from John Donne

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

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

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

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

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

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

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

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

Abstract ...................................................................................................................... i  
Acknowledgements ..................................................................................................... iii  
Abbreviations ................................................................................................................. x  

**Chapter 1** Introduction ........................................................................................................ 1  
  1.1 Translational Control ............................................................................................. 2  
  1.2 Role of eIF2α in translational initiation ................................................................. 3  
  1.3 Selective translation mediated by eIF2α phosphorylation ..................................... 5  
  1.4 Mechanism of augmented GCN4 mRNA translation .......................................... 7  
  1.5 eIF2α kinase: Gcn2 .............................................................................................. 9  
  1.6 Effector proteins Gcn1 and Gcn20 ......................................................................... 9  
  1.7 Role of Gcn1 in activation of Gcn2 ..................................................................... 11  
  1.8 Translational elongation ..................................................................................... 13  
  1.9 Fungal elongation factor 3 (eEF3) ....................................................................... 14  
  1.10 Eukaryotic elongation factor 1 A (eEF1A) ......................................................... 16  
  1.11 Aims and objectives ......................................................................................... 18  
  1.12 Significance of the study ............................................................................... 18  

**Chapter 2** Materials and Methods .................................................................................... 20  
  2.1 Biological materials ............................................................................................. 21  
  2.2 Plasmid constructions .......................................................................................... 21  
    2.2.1 pJV01 ........................................................................................................ 21  
    2.2.2 pJV02 ........................................................................................................ 21  
  2.3 Media ................................................................................................................. 24  
    2.3.1 Bacterial media ......................................................................................... 24  
    2.3.2 Yeast media .............................................................................................. 24  
    2.3.3 Media supplements ............................................................................... 25  
  2.4 Growth conditions ............................................................................................. 26  
    2.4.1 Bacterial growth conditions .................................................................... 26  
    2.4.2 Yeast growth conditions ....................................................................... 26  
  2.5 Glycerol stocks .................................................................................................... 26  
  2.6 DNA isolation and purification ......................................................................... 26  
    2.6.1 Plasmid DNA isolation and purification ................................................. 26  
    2.6.2 Genomic DNA isolation ................................................................. 28  
  2.7 DNA quantification ............................................................................................. 28  
  2.8 Agarose gel electrophoresis ............................................................................... 29
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9 Restriction endonuclease digestion</td>
<td>29</td>
</tr>
<tr>
<td>2.10 Polymerase Chain Reaction</td>
<td>29</td>
</tr>
<tr>
<td>2.10.1 Standard PCR</td>
<td>29</td>
</tr>
<tr>
<td>2.10.2 Colony PCR</td>
<td>30</td>
</tr>
<tr>
<td>2.10.3 dNTPs</td>
<td>30</td>
</tr>
<tr>
<td>2.10.4 Primers</td>
<td>30</td>
</tr>
<tr>
<td>2.11 DNA purification</td>
<td>32</td>
</tr>
<tr>
<td>2.11.1 Purification of PCR products using QIAquick PCR Purification Kit</td>
<td>32</td>
</tr>
<tr>
<td>2.11.2 Extraction and purification of DNA from agarose gels using</td>
<td>32</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit (Qiagen)</td>
<td></td>
</tr>
<tr>
<td>2.12 DNA ligations</td>
<td>32</td>
</tr>
<tr>
<td>2.12.1 Dephosphorylation</td>
<td>32</td>
</tr>
<tr>
<td>2.12.2 Ligations</td>
<td>32</td>
</tr>
<tr>
<td>2.13 Transformation of E. Coli</td>
<td>33</td>
</tr>
<tr>
<td>2.13.1 Preparation of competent E. coli using CaCl₂</td>
<td>33</td>
</tr>
<tr>
<td>2.13.2 Transformation of E. coli by heat shock</td>
<td>33</td>
</tr>
<tr>
<td>2.14 DNA sequencing</td>
<td>33</td>
</tr>
<tr>
<td>2.15 Yeast transformation</td>
<td>34</td>
</tr>
<tr>
<td>2.15.1 Making Yeast Competent</td>
<td>34</td>
</tr>
<tr>
<td>2.15.2 Transformation</td>
<td>34</td>
</tr>
<tr>
<td>2.16 Preparation of Whole Cell Extract (WCE)</td>
<td>34</td>
</tr>
<tr>
<td>2.16.1 Standard WCE preparation</td>
<td>34</td>
</tr>
<tr>
<td>2.16.2 Formaldehyde cross-linked WCE preparation</td>
<td>35</td>
</tr>
<tr>
<td>2.17 Estimation of protein concentration</td>
<td>36</td>
</tr>
<tr>
<td>2.17.1 Estimation by Bradford method</td>
<td>36</td>
</tr>
<tr>
<td>2.17.2 Estimation by absorbance under UV</td>
<td>37</td>
</tr>
<tr>
<td>2.18 Ribosome co-sedimentation</td>
<td>37</td>
</tr>
<tr>
<td>2.19 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)</td>
<td>37</td>
</tr>
<tr>
<td>2.19.1 Gradient gel electrophoresis</td>
<td>37</td>
</tr>
<tr>
<td>2.19.2 Discontinuous gel electrophoresis</td>
<td>38</td>
</tr>
<tr>
<td>2.20 Staining proteins in acrylamide gels</td>
<td>39</td>
</tr>
<tr>
<td>2.21 Western Blotting</td>
<td>39</td>
</tr>
<tr>
<td>2.21.1 Gel Transfer</td>
<td>39</td>
</tr>
<tr>
<td>2.21.2 Staining proteins on membranes</td>
<td>39</td>
</tr>
<tr>
<td>2.22 Immunological detection of proteins</td>
<td>40</td>
</tr>
</tbody>
</table>
A.2 Calculation of relative levels of eIF2α-P from raw data ......................................122
A.3 eEF3 over-expression barely affects Gcn1-polysome association .......................123
A.4 stm1Δ strain is SM resistant ..............................................................................126
Appendix B .................................................................................................................... ...127
B.1 Verification of pJV02 .........................................................................................128
B.2 Gcn2 co-elutes with eEF1A under replete conditions ........................................128
B.3 Gcn2-eEF1A interaction is lost upon amino acid starvation. .........................129
References .................................................................................................................... ...130

List of figures

Figure 1.1: Representation of protein kinases being activated by different stress conditions to regulate the rate of protein synthesis via eIF2α phosphorylation. (Adapted from (Wek et al., 2006))................................................................. 3
Figure 1.2: Schematic representation of translation initiation. ................................. 4
Figure 1.3: Order of molecular events in the general amino acid control (GAAC). .... 6
Figure 1.4: Representation of GCN4 translation during (A) replete and (B) starved conditions............................................................................................................... 8
Figure 1.5: Schematic representation of domains in Gcn2............................... 9
Figure 1.6: Representation of the domains in Gcn1 and Gcn20. The domains are juxtaposed when in a Gcn1/20 complex, resembling the fungal Elongation Factor 3 (eEF3) .................................................................................................................... 10
Figure 1.7: Model for Gcn1 mediated amino acid depletion sensing and activation of Gcn2 by deacylated tRNA- (Sattlegger and Hinnebusch, 2000) ......................... 13
Figure 1.8: (A) Representation of domains in eEF3, (B) Crystal structure of eEF3- ADP taken from (Andersen et al., 2006).............................................................................. 16
Figure 3.1: Protein expression detectable by colony western ................................. 47
Figure 3.2: Colony western is a semi-quantitative assay ......................................... 48
Figure 3.3: Untagged proteins can be detected by colony western ....................... 49
Figure 3.4: Colony westerns can be used to determine the optimal concentration of inducing agent for protein expression ................................................................. 51
Figure 4.1: Over-expression of eEF3 causes sensitivity to amino acid analogue 3AT 57
Figure 4.2: The slg phenotype associated with GCN2Δ can be reverted by galactose induced over-expression of eEF3 ........................................................................... 58
Figure 4.3: Over-expression of eEF3 reduces eIF2 phosphorylation by Gcn2 under starvation. .......................................................................................................................... 60
Figure 4.4: eEF3 over-expression impairs eIF2 phosphorylation by Gcn2 upon starvation. .......................................................................................................................... 62
Figure 4.5: eEF3 over-expression barely displaces Gcn1 from the polyribosomes .. 65
Figure 4.6: Percentage distribution of (A) Gcn1 and (B) Gcn2 in fractions collected in Figure 4.5 ............................................................................................................... 66
Figure 4.7: Percentage distribution of Gcn1 in the different fractions. .................. 68
Figure 4.8: Percentage distribution of Gcn2 in the different fractions. .................. 69
Figure 4.9: The eEF3 C-terminus is sufficient for causing a Gcn\(^-\) phenotype. .... 72
Figure 4.10: The eEF3 C-terminus is necessary for causing a Gcn\(^-\) phenotype .... 74
Figure 4.11: The eEF3 C-terminus co-migrates with polysomes ......................... 76
Figure 4.12: Positions of eEF3 and Gcn1 on the small ribosomal subunit .......... 82
Figure 4.13: Model for exclusive ribosome function of eEF3 and Gcn1 .......... 83
Figure 5.1: Gcn2 co-elutes with His\(_6\)-eEF1A ......................................................... 88
Figure 5.2: Gcn2 co-immunoprecipitates with eEF1A ......................................... 89
Figure 5.3: GST-Gcn2-C terminus interacts with eEF1A and ribosomes ............ 90
Figure 5.4: eEF1A-Gcn2 interaction is not mediated via the ribosome .......... 92
Figure 5.5: Comparison of the amount of eEF1A and ribosomal proteins bound to Gcn2 CTD and the Gcn2 CTD K3 .......................................................................................... 93
Figure 5.6: eEF1A-Gcn2 interaction is independent the ribosome ....................... 95
Figure 5.7: Coomassie stained gel with purified eEF1A ....................................... 96
Figure 5.8: Direct interaction between eEF1A and Gcn2 ..................................... 97
Figure 5.9: Amount of eEF1A bound to the Gcn2 fragments ................................ 97
Figure 5.10: eEF1A-Gcn2 interaction is not bridged by RNA ................................. 98
Figure 5.11: Similar amounts of eEF1A bound the Gcn2 fragments with or without RNase digestion ........................................................................................................... 99
Figure 5.12: eEF1A-Gcn2 interaction not detectable upon amino acid starvation .. 101
Figure 5.13: Histidine starvation is detected within 1 min after the addition of 3AT ................................................................................................................................. 103
Figure 5.14: Reduction in growth temperature delays eIF2\(\alpha\) phosphorylation ....... 104
Figure 5.15: eIF2\(\alpha\)-P levels over a duration of different starvation times .......... 105
Figure 5.16: General protein synthesis is reduced within 2 minutes of adding 3AT at a growth temperature of 30°C .................................................................107
Figure 5.17: Reduction in translation takes 2 minutes after addition of 3AT at a growth temperature of 25°C .................................................................................108
Figure 5.18: Reduction in translation takes 4 minutes after addition of 3AT at a growth temperature of 20°C .................................................................................109
Figure 5.19: Model for eEF1A as an inhibitor of Gcn2 ............................................114
Figure 6.1: Model for translation elongation regulating translational control ........119
Figure A.1: Verification of pJV01 by restriction digestion ........................................121
Figure A.2: eEF3 over-expression barley displaced Gcn1 from the polyribosomes .124
Figure A.3: eEF3 over-expression barley displaced Gcn1 from the polyribosomes .125
Figure A.4: $stm1\Delta$ strain is SM resistant .............................................................126
Figure B.1: Gcn2 co-elutes with eEF1A ..................................................................129
Figure B.2: Gcn2-eEF1A interaction lost upon amino acid starvation .................129

List of tables

Table 2.1: Yeast Strains used in this study ..............................................................22
Table 2.2: Plasmids used in this study .................................................................23
Table 2.3: List of antibiotics used .......................................................................25
Table 2.4: List of induction drugs used ...............................................................25
Table 2.5: List of amino acids used .....................................................................25
Table 2.6: List of primers used ............................................................................31
Table 2.7: List of primary antibodies and dilutions used in this study ...............41
Table 2.8: List of secondary antibodies and dilutions used in this study ..........41
Abbreviations

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

3AT    3 amino triazole  
aa    amino acid  
aa-tRNA    amino acylated tRNA  
ABC    ATP-binding cassette  
APS    ammonium persulphate  
A-site    acceptor-site  
ATP    adenosine triphosphate  
bp    base pair  
BSA    Bovine Serum Albumin  
CIA    chloroform: iso-amyl alcohol  
CIP    calf intestinal phosphatase  
CTD    C-terminal domain  
DNA    deoxyribonucleic acid  
dNTP    deoxyribonucleotide triphosphate  
DTT    dithiothreitol  
EDTA    ethylenediamine tetra acetic acid  
eEF1A    eukaryotic elongation factor 1A  
eEF3    eukaryotic elongation factor 3  
EF2    elongation factor 2  
eIF2    eukaryotic initiation factor 2  
E-site    exit-site  
GAAC    general amino acid control  
Gcn    general control non derepressible  
GDP    guanosine diphosphate  
GTP    guanosine triphosphate  
HEPES    4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid  
His-RS    histidyl-tRNA synthetase  
IPTG    isopropyl-β-D-thiogalactopyranoside  
LB    luria-bertani  
Ni-NTA    nickel-nitrilo triacetic acid  
OD    optical density  
ORF    open reading frame  
p    plasmid  
PAGE    polyacrylamide gel electrophoresis  
PCR    polymerase chain reaction  
PEG    polyethylene glycol  
PMSF    phenylmethanesulphonyl fluoride  
PRS    post ribosomal supernatent  
P-site    peptidyl donor site  
PVDF    polyvinylidene difluoride  
rpm    revolutions per minute  
RNase    ribonuclease  
RWD    RING finger proteins, WD-repeat-containing proteins, yeast DEAD-like helicases  
SD    synthetic dropout  
SDS    sodium dodecyl sulfate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM</td>
<td>sulfometuron methyl</td>
</tr>
<tr>
<td>tRNA&lt;sub&gt;Met&lt;/sub&gt;</td>
<td>Methionyl-tRNA</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>TBS-Tween</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TOR</td>
<td>target of rapamycin</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>WCE</td>
<td>whole cell extract</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
</tbody>
</table>