

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.

Identification of potential Gcn2 regulating proteins

A thesis presented in partial fulfilment of the requirements for the degree of
Master of Science
in Genetics

at Massey University, Albany, New Zealand

Hayley Dawn Prescott

2016

Abstract

The viability of any organism relies greatly on their ability to adapt their metabolic processes in response to environmental stimuli. Proteins are essential for almost every intracellular biological process. Proteins are composed of amino acid precursors and the levels of amino acids within the cell available for protein synthesis requires careful monitoring. Amino acid conservation needs to be initiated in response to declining availability. Under conditions of amino acid deprivation, cessation of non-essential protein synthesis and activation of amino acid biosynthetic pathways is initiated. This regulatory mechanism is referred to as the General Amino Acid Control (GAAC) pathway.

General control non-derepressible 2 (Gcn2) is a fundamental constituent of the GAAC response pathway. Gcn2 senses a decline in amino acid availability and initiates the stress response by phosphorylating the alpha subunit of eukaryotic translation initiation factor 2 (eIF2 α). The phosphorylation of eIF2 α triggers a sequence of events resulting in increased translation of the transcriptional activator Gcn4, which subsequently induces selective expression of genes necessary for *de novo* amino acid synthesis.

The activation and activity of Gcn2 is moderated by inhibitory and facilitative protein interactions. Published large scale purification studies identified many novel Gcn2 binding partners, some of which may function in Gcn2 regulation. However known Gcn2 regulators were absent from these datasets indicating that they were incomplete.

This work aimed to identify and screen potential Gcn2 binding partners for those that regulate Gcn2 activity. Analysis identified 135 proteins that were potentially in complex with Gcn2. Of those, Sse1, Chs5, Ncl1, Tir4 and Npr1 were subsequently identified as potential Gcn2 regulators.

For the purpose of comprehensively identifying novel Gcn2 binding proteins, a protocol was successfully optimised to enable Gcn2 affinity purification under conditions that would be specifically conducive for the maintenance of bonds between Gcn2 and its interaction partners. In this method, Gcn2 was overexpressed in cells to drive weak and/or transient interactions, and the usage of formaldehyde to crosslink interactions and further stabilise them was explored. This method will enable the future compilation of a comprehensive Gcn2 interactome.

Acknowledgements

In this space I would like to acknowledge those people who have contributed to the content of this thesis.

I am extremely grateful to my supervisor Evelyn Sattlegger. I would like to thank you for your support, advice and encouragement during the course of this research. You have been an incredible mentor to me.

I would like to thank my laboratory colleagues and friends Rashmi Ramesh and Kayleigh Evans who have always been on hand to offer advice and encouragement, and for proof reading my thesis for me.

I would also very grateful to Renuka Shanmugam for her incredibly valuable technical advice and encouragement.

Last of all I would like to thank my family, who have supported me wholeheartedly throughout the course of my studies.

Table of contents

| | |
|---|------|
| Abstract..... | I |
| Acknowledgement..... | II |
| Table of contents..... | III |
| List of Figures..... | VI |
| List of Tables..... | VIII |
| Abbreviations..... | IX |
| | |
| Introduction..... | 1 |
| 1.1.1 The General Amino Acid Control Pathway (GAAC)..... | 2 |
| 1.1.2 Gcn2 phosphorylates eIF2 α to reduce the rate of protein translation initiation..... | 3 |
| 1.1.3 Gcn2 activation and eIF2 phosphorylation promotes de-repression of Gcn4..... | 4 |
| 1.1.4 The GAAC pathway is conserved among all eukaryotic lineages..... | 6 |
| 1.1.5 The structure of Gcn2..... | 7 |
| 1.1.6 Proteins that bind and regulate Gcn2..... | 8 |
| 1.1.7 The diverse functions of Gcn2..... | 10 |
| 1.2 Hypothesis and aim of this research..... | 12 |
| | |
| Materials and Methods..... | 13 |
| 2.1 Biological Materials..... | 14 |
| 2.2 Media..... | 18 |
| 2.3 Media supplements..... | 19 |
| 2.4 Media Preparation..... | 20 |
| 2.5 Growth conditions..... | 20 |
| 2.6 Long term storage of Yeast and bacterial strains..... | 21 |
| 2.7 Measuring optical density..... | 21 |
| 2.8 Semi quantitative growth assay..... | 21 |
| 2.9 Formaldehyde crosslinking..... | 21 |
| 2.10 Mechanical cell lysis for whole cell extract preparation..... | 22 |

| | |
|--|----|
| 2.11 Bradford protein assay | 22 |
| 2.12 Yeast whole cell extract preparation using chemical lysis | 23 |
| 2.13 Plasmid amplification and purification | 23 |
| 2.14 Agarose Gel Electrophoresis | 24 |
| 2.15 Yeast transformation | 24 |
| 2.16 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) | 25 |
| 2.17 Western blotting | 26 |
| | |
| Identification of potential Gcn2 regulating proteins | 28 |
| 3.1 Identification of putative Gcn2 binding proteins | 29 |
| 3.1.1 Gcn2 Primary Interactome | 37 |
| 3.1.2 Gcn2 Secondary Interactome | 38 |
| 3.1.3 Identification of proteins that potentially form a complex with Gcn2 | 41 |
| 3.2 Identification of potential Gcn2 regulating proteins | 44 |
| 3.2.1 Screen of single gene deletion mutants for sensitivity to sulfometuron methyl | 44 |
| 3.2.2 Screening SMs strains for those with impaired Gcn2 function | 51 |
| 3.2.3 Validation that SMs is the result of the single gene deletion | 55 |
| 3.3 Screening putative Gcn2 binding proteins to identify inhibitors of Gcn2 activity or activation | 57 |
| 3.3.1 Screening single gene overexpression strains for sensitivity to Sulfometuron Methyl | 57 |
| 3.3.2 Screening overexpression strains with SMs for impaired Gcn2 function | 59 |
| 3.4 Establishment of a his ₆ tag mediated Gcn2 affinity purification procedure | 61 |
| 3.4.1 Verification of Gcn2 expression | 62 |
| 3.4.2 Optimisation of Gcn2 purification using iMAC resin | 65 |
| 3.4.3 Formaldehyde crosslinking optimisation | 69 |
| 3.4.4 Protease inhibitor composition does not reduce Gcn2 degradation | 73 |
| 3.4.5 Gcn2 iMAC purification with Imidazole elution | 74 |
| 3.4.6 Large scale purification of Gcn2 and bound interaction partners | 75 |

| | |
|--|-----|
| Discussion..... | 78 |
| 4.1 The identification of potential Gcn2 binding proteins..... | 79 |
| 4.1.1 The interactome analysis did not identify all of the known Gcn2 binding proteins..... | 82 |
| 4.1.2 Proteins identified in the primary Gcn2 interactome analysis as both bait and prey..... | 84 |
| 4.1.3 Identification of potential complexes that contain Gcn2..... | 87 |
| 4.1.4 Identification of new regulators that promote Gcn2 activity..... | 88 |
| 4.1.5 Screening for potential negative regulators of Gcn2..... | 91 |
| 4.2 Establishment of a his ₆ tag mediated Gcn2 affinity purification procedure..... | 94 |
| 4.3 Conclusion and future directions | 97 |
| Appendix..... | 98 |
| Appendix 1..... | 99 |
| Appendix 2..... | 104 |
| Appendix 3..... | 106 |
| References..... | 107 |

List of Figures

| | |
|---|----|
| Figure 1.1 Schematic of differential GCN4 translation under replete and amino acid starved conditions..... | 5 |
| Figure 1.2 Schematic of Mammalian eIF2 α kinases. Mammals have four distinct eIF2 α kinases that are activated in response to a specific cellular stress condition... | 6 |
| Figure 1.3 Schematic overview of the five characterised domains of Gcn2..... | 8 |
| Figure 3.0 Schematic of Affinity Purification principle..... | 26 |
| Figure 3.1 Overview of FLAG mediated affinity purification..... | 33 |
| Figure 3.2 Overview of tandem affinity purification method using the TAP tag epitope..... | 34 |
| Figure 3.3 Schematic of Gcn2 interactome data analysis approach..... | 36 |
| Figure 3.4 Gcn2 primary interactome..... | 38 |
| Figure 3.5 Gcn2 secondary interactome..... | 40 |
| Figure 3.6 Identification of a potential protein complex that includes Gcn2, Cdc48 and Shp..... | 41 |
| Figure 3.7 Identification of a potential protein complex that includes Gcn2, Tba1, Hca4 and Gdc11..... | 42 |
| Figure 3.8 Identification of a potential protein complex that includes Gcn2, Pph3 and Psy2..... | 43 |
| Figure 3.9 Single gene deletion mutants that displayed sensitivity to sulfometuron methyl..... | 46 |
| Figure 3.10 eIF2 α -P levels in mutants that display sensitivity to SM..... | 52 |
| Figure 3.11 comparison of eIF2 α -p levels in cultures grown under starved and replete conditions..... | 53 |
| Figure 3.12 Tiling collection plasmid inserts containing genes of interest..... | 56 |
| Figure 3.13 Screening to identify proteins that when overexpressed render cells SMs..... | 58 |
| Figure 3.14 comparison of eIF2 α -p levels in starved and replete cultures of SMs gene overexpression strains with the wild type | 60 |
| Figure 3.15 Determining the Gcn2 expression levels in yeast cells..... | 63 |
| Figure 3.16 Gcn2 is detectable in extracts from Gcn2-overexpression strains generated by mechanical lysis..... | 64 |
| Figure 3.17 Purification of Gcn2 from cell extract..... | 66 |

| | |
|--|----|
| Figure 3.18 Attempted Gcn2 purification using formaldehyde crosslinked samples and imidazole elution..... | 67 |
| Figure 3.19 Immunoblot wash buffer from unsuccessful pull-down assay..... | 68 |
| Figure 3.20 Representation of typical results from iMAC pull-down assays for Gcn2 purification..... | 69 |
| Figure 3.21 Formaldehyde crosslinking optimisation..... | 71 |
| Figure 3.22 Purification of Gcn2 from cell extract..... | 72 |
| Figure 3.23 Comparison of Gcn2 purifications performed in presence of different protease inhibitor compositions..... | 74 |
| Figure 3.24 iMAC mediated Gcn2 purification with imidazole elution..... | 75 |
| Figure 3.25 Large scale iMAC mediated purification of Gcn2..... | 77 |

List of Tables

| | |
|--|----|
| Table 2.1 <i>Saccharomyces cerevisiae</i> strains used in this study..... | 14 |
| Table 2.2 Plasmids used in this study | 16 |
| Table 2.3 Amino acid media supplements..... | 19 |
| Table 2.4 Antibiotics and starvation induction drugs | 19 |
| Table 3.1 Comparison of large scale Affinity Purification Mass Spectrometry protein interaction studies..... | 30 |
| Table 3.2 Final SM sensitivity scores for all deletion strains subjected to semi-quantitative growth assays..... | 49 |
| Table 3.3 Summary eIF2-P levels SMs mutant strains..... | 54 |
| Table 4.1. Comparison of lysis buffer components used in large scale AP-MS studies | 81 |

Abbreviations

| | |
|------------------|---|
| AT | 3-Amino-1, 2, 4-triazole |
| APS | Ammonium persulfate |
| A-site | Acceptor site |
| BSA | Bovine serum albumin |
| Co-IP | Co-immunoprecipitation |
| EDTA | Ethylenediaminetetraacetic acid |
| eEF1A | Eukaryotic translation elongation factor 1 A |
| eIF2 | Eukaryotic initiation factor 2 |
| eIF2 α -P | Eukaryotic initiation factor 2 alpha phosphorylated |
| EtBr | Ethidium Bromide |
| GAAC | General amino acid control |
| Gcn1 | General control non-derepressible 1 |
| Gcn2 | General control non-derepressible 2 |
| Gcn4 | General control non-derepressible 4 |
| HCl | Hydrochloric acid |
| kDa | Kilo Dalton |
| LiOAc | Lithium Acetate |
| mRNA | Messenger ribonucleic acid |
| NaCl | Sodium Chloride |
| NaOH | Sodium hydroxide |
| ORF | Open reading frame |
| PEG | Polyethylene glycol |
| Pgk1 | Phosphoglycerate kinase 1 |

| | |
|------|--------------------------------|
| PMSF | Phenylmethanesulfonyl fluoride |
| PVDF | Polyvinylidene fluoride |
| rpm | Revolutions per minute |
| SD | Synthetic dextrose |
| SDS | Sodium dodecyl sulfate |
| SM | Sulfometuron methyl |
| TBS | Tris Buffered Saline |
| TBST | Tris buffered Saline Tween |
| TC | Ternary complex |
| YPD | Yeast peptone dextrose |
| YPG | Yeast peptone glycerol |