Identification of potential Gcn2 regulating proteins

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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 (eIF2α). The phosphorylation of eIF2α 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.

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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
eIF2α-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

Chapter 1

Introduction

1.1 The General Amino Acid Control Pathway (GAAC)

All organisms are subject to environments which are dynamic and unpredictable. The viability of an organism is in part due to their ability to adapt to both external and internal stimuli that may cause cellular stress. Most heterotrophic organisms will not continuously intake, or have access to, macronutrients and will consequently experience periods of nutritional deficiency. Mechanisms have therefore evolved in these organisms to regulate metabolic processes to maintain cellular homeostasis (Brobeck, 1948). Proteins are essential macromolecules that actively contribute to almost every function performed by a living organism. The ability to synthesize proteins is an essential requisite for survival and this requires a sufficient pool of amino acids, the basic constituents of proteins. The pool of available amino acids needs to be monitored and if the supplies decrease, then amino acid conservation needs to be initiated. The strategy for this regulation is referred to as the General Amino Acid Control (GAAC) pathway (Delforge, Messenguy, & Wiame, 1975). This mechanism has developed to respond to the specific stress of amino acid deprivation and has been conserved among all eukaryotic lineages. The GAAC pathway can be triggered by starvation for even a single amino acid (A. G. Hinnebusch, 2005). When this occurs, processes are initiated to reduce global protein synthesis conserving precious amino acid resources, and gene expression patterns are modified to increase the rate of amino acid biosynthesis (Hinnebusch, 2005). One of the key components of the GAAC pathway is General control non-derepressible 2 (Gcn2). Gcn2 activation under conditions of amino acid deprivation, enables organisms to overcome the nutrient deficiency for an extended period of time (Hinnebusch, 2005).

1.1.2 Gcn2 phosphorylates eIF2α to reduce the rate of protein translation initiation

The translation of protein from mRNA in eukaryotic organisms is initiated by eukaryotic initiation factor 2 (eIF2). In active form, eIF2 is bound to guanosine triphosphate (GTP), this complex then binds methionyl-tRNA (Met-$tRNA_i^{Met}$) to form the eIF2-GTP-Met-$tRNA_i^{Met}$ ternary complex (TC) (A. G. Hinnebusch, 2000). The TC then binds to the 40S ribosome subunit to generate a 43s pre-initiation complex (Asano, Clayton, Shalev, & Hinnebusch, 2000a). This complex associates with the 5' cap of an mRNA, and proceeds in the 3' direction, scanning for the AUG start codon. Once base pair recognition occurs between the methionine coding AUG and the $tRNA_i^{Met}$ anti-codon (Asano, Clayton, Shalev, & Hinnebusch, 2000b), eIF2-GTP is hydrolysed to inorganic phosphate (Pi) and eIF2-GDP, eliciting its release from the ribosome. The large ribosomal subunit is then recruited, and the complete functional ribosome can commence the elongation phase of translation. Translation initiation is a cyclic process, eIF2 is released from the initiation complex bound to GDP as an inactive binary complex (Kimball, 1999). To resume its active form, eIF2-GDP must exchange GDP for GTP. As the affinity of eIF2 for GDP is higher than its affinity for GTP, the reaction requires catalysis by eIF2B, the eIF2 guanine nucleotide exchange factor (A. G. Hinnebusch, 2000; Rowlands, Panniers, & Henshaw, 1988). The active eIF2-GTP can then bind $tRNA_i^{Met}$ and commence another translation initiation cycle.

When the nutritional requirements of an organism are satiated, the synthesis of proteins occurs continuously from a replete supply of amino acid precursors. When amino acid supplies become limited, there is a corresponding increase in the amount of tRNA in the cell that is not bound to an amino acid. Gcn2 senses the amino acid limitation by detecting the deacylated tRNAs accumulating within the cell, and becomes activated (J. Dong, H. Qiu, M. Garcia-Barrio, J. Anderson, & A. G. Hinnebusch, 2000). Gcn2 is a kinase that, when activated, phosphorylates serine 51 of the alpha subunit of eIF2. Phosphorylation of eIF2α promotes strong binding of eIF2B to eIF2α-P-GDP, this obstructs the cycling of the inactive eIF2-GDP to its active eIF2-GTP state (T. Krishnamoorthy, Pavitt, Zhang, Dever, & Hinnebusch, 2001). The consequence of this is a reduction in the amount of ternary complex in the cell, decreased Met-$tRNA_i^{Met}$ delivery to the ribosome, and reduced global protein synthesis.

1.1.3 Gcn2 activation and eIF2 phosphorylation promotes de-repression of Gcn4

During amino acid starvation, the activation of Gcn2 promotes amino acid preservation, significantly reducing cellular global protein translation levels, as described in the previous chapter. However, another crucial aspect of the GAAC response is the increased expression of genes which code for protein products that are necessary for amino acid biosynthesis to overcome starvation, such as Gcn4. Gcn4 is an essential component of the transcriptional response to amino acid starvation that positively regulates the transcription of amino acid biosynthetic genes (Alan G. Hinnebusch & Fink, 1986). The regulation of Gcn4 itself occurs at the point of mRNA translation (A. G. Hinnebusch, 1984, 1997; A. G. Hinnebusch, Jackson, & Mueller, 1988). Under replete conditions, Gcn4 is translated at a low basal level. This is due to the presence of four short upstream Open Reading Frames (uORFs) in the leader sequence of the Gcn4 mRNA.

Under replete conditions, the 43s pre-initiation complex binds to the 5' terminus of the mRNA and proceeds to scan the sequence until an AUG start codon is recognised. Upon start codon recognition, the large ribosomal subunit (60S) is recruited, the functional ribosome assembles and translation ensues. Following termination of translation of the ORF, the large ribosomal subunit dissociates from the mRNA. However most of the 40S ribosomal subunits remain associated with the mRNA and continue to scan the mRNA for subsequent start codons. During the scanning process the 40S subunit re-acquires a ternary complex (TC), allowing it to recognize the AUG start codon of ORF 2, 3, or 4, where translation will again be initiated (Kozak, 1989).

Following translation of uORFs 2, 3 or 4, the majority of ribosomes will dissociate from the mRNA before the *GCN4* start codon is encountered (Figure1.1), effectively repressing *GCN4* translation (Grant, Miller, & Hinnebusch, 1994; A. G. Hinnebusch, 1993).

Under starvation conditions, there is translation of uORF 1, but the ribosome is less likely to reinitiate translation at the start codons of uORFs 2, 3 and 4. This is due to the amount of ternary complex within the cell being significantly reduced. Under this condition, the time it takes to scan from ORF1 to the other ORFs is too short to re-acquire a ternary complex. Without a ternary complex, the ribosome is not able to recognize start codons and instead continues to scan past the ORFs (Figure1.1).

Although uORFs 1, 2, 3 and 4 are situated in close proximity to each other, there is a substantial distance between uORF4 and *GCN4*. The increased distance that the ribosome must scan before encountering the *GCN4* start codon provides the ribosome with a sufficient timeframe to bind a ternary complex for re-initiation of translation at *GCN4* (A. G. Hinnebusch, 1993; Kozak, 1987).

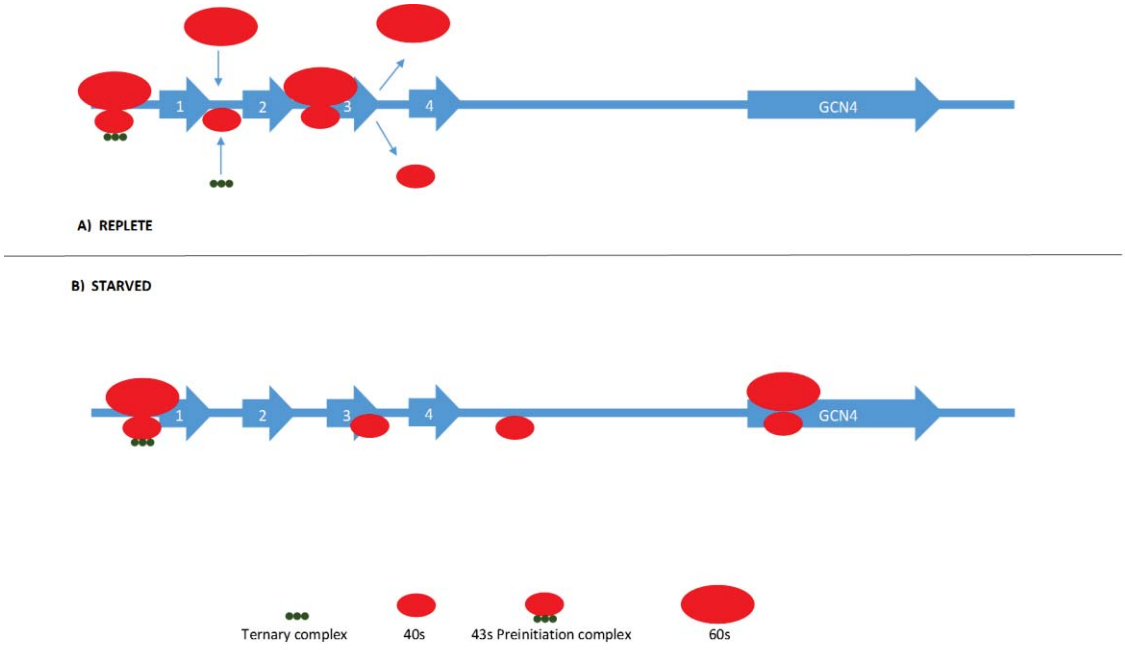


Figure 1.1 Schematic of differential GCN4 translation under replete and amino acid starved conditions. A) Under replete conditions the 43s preinitiation complex binds to the 5' terminus of the GCN4 mRNA and translates the uORF1 and then one or more of the other three ORFs (labelled 1 to 4). Most ribosomes (red) will dissociate from the mRNA before they reach GCN4. **B)** Under starvation conditions, there is translation of uORF1. However due to deplete TC levels in the cell, the majority of ribosomes will scan past the ORFs 2 to 4 and reinitiate translation at the GCN4 reading frame. For more detail see above text

1.1.4 The GAAC pathway is conserved among all eukaryotic lineages

Gcn2 is the only eIF2α kinase present in the budding yeast *Saccharomyces cerevisiae* and is highly conserved among all eukaryotes (Castilho et al., 2014). Mammals have an additional three eIF2α kinases that are each activated under a specific cellular stress condition. Protein kinase double-stranded RNA-dependent (PKR) induces protein synthesis arrest in response to viral infection, consequentially disrupting viral replication (Galluzzi, Brenner, Morselli, Touat, & Kroemer, 2008; Michael, 1996). PKR-like endoplasmic reticulum kinase (PERK) is activated by endoplasmic reticulum stress caused by amassing incorrectly folded proteins which are toxic to the cell (Berlanga et al., 2006; Kaufman, 2002; Shi et al., 1998; Szegezdi, Logue, Gorman, & Samali, 2006). Heme-regulated inhibitor (HRI) is found in red blood cells. It phosphorylates eIF2α in response to the unavailability of free heme molecules. That way it adjusts globin synthesis to the availability of heme molecules and prevents the cellular accumulation of globin chains (J.-J. Chen, 2007; de Haro, Mendez, & Santoyo, 1996; Yun, Matts, & Matts, 2005). In humans, the phosphorylation of eIF2α by these four kinases and subsequent translation of transcriptional activator Atf4 is known as the Integrated Stress Response (ISR) pathway (Harding et al., 2003). The conservation of Gcn2 among all eukaryotic organisms emphasises how significant the strategy of amino acid control is for organism viability. The fact that Gcn2 is highly conserved from yeast to humans, facilitates efficient investigation into general Gcn2 regulation, using yeast as a model organism.

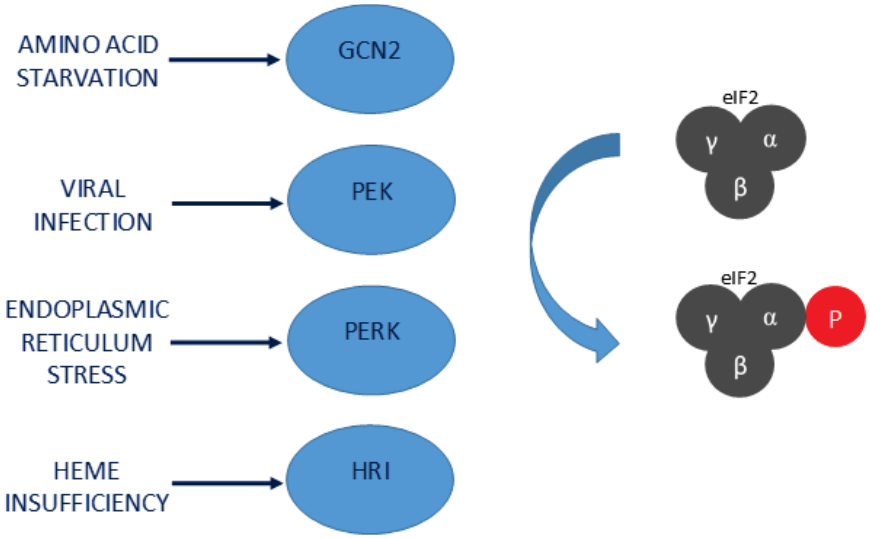


Figure 1.2 Schematic of Mammalian eIF2α kinases. Mammals have four distinct eIF2α kinases that are activated in response to a specific cellular stress condition.

1.1.5 The structure of Gcn2

The serine/threonine kinase Gcn2 is composed of multiple domains, five of these domains have been characterised. The N-terminus contains the RWD domain, named due to the presence of RING finger proteins, WD-repeat-containing proteins, and yeast DEAD-like helicase homologous sequences. This domain houses a binding site for Gcn1, a key Gcn2 effector protein (Sattlegger & Hinnebusch, 2000b). Adjacent to the RWD domain is a pseudo kinase domain (ψPK) which is structurally similar to a functional kinase domain, however, it lacks two motifs required for catalytic activity, the Val-Ala-Ile-Lys (VAIK) motif and the His-Arg-Asp (HRD) motif, necessary for ATP interaction and proton transfer respectively (Lageix, Zhang, Rothenburg, & Hinnebusch, 2015). The protein kinase (PK) domain is situated in between the ψPK domain and the Histidyl tRNA synthetase-like (HisRS) domain, and is the location of the protein kinase catalytic activity.

The HisRS domain, together with the Gcn2 C-terminal domain, is the region that binds the starvation signal, uncharged tRNAs, and through conformational changes triggers activation of the protein kinase domain (Qiu, Dong, Hu, Francklyn, & Hinnebusch, 2001a; Wek, Jackson, & Hinnebusch, 1989b). The HisRS domain has a high level of similarity to histidyl-tRNA synthetases that have a motif 2, which is essential for binding tRNA (Ruff et al., 1991). In contrast to His-tRNA synthetases the HisRS domain in Gcn2 binds only $tRNA^{deacyl}$ and does not charge the tRNA with amino acids (J. Dong et al., 2000). Mutations introduced to motif 2 (m2 mutation) of the HisRS domain prevents the binding of tRNA and as a result the Gcn2 protein kinase function ceases (S. A. Wek, S. H. Zhu, & R. C. Wek, 1995; Zhu, Sobolev, & Wek, 1996). Although the domain is homologous to histidyl-tRNA synthetases, binding is not restricted to $tRNA^{His}$, Gcn2 kinase activity is also stimulated by other uncharged tRNA species binding to the HisRS domain (J. Dong et al., 2000).

Flanking the HisRS region is the protein C-terminal domain which contains a lysine rich region. Three lysine residues in particular, K1552, K1553 and K1556, are conserved and necessary for $tRNA^{deacyl}$ binding. These three residues are also implicated in binding of Gcn2 to the ribosome. Deletion and substitutions of the lysine residues results in Gcn2 being unable to bind tRNA and become activated (J. S. Dong, H. F. Qiu, M. Garcia-Barrio, J. Anderson, & A. G. Hinnebusch, 2000; Zhu & Wek, 1998).

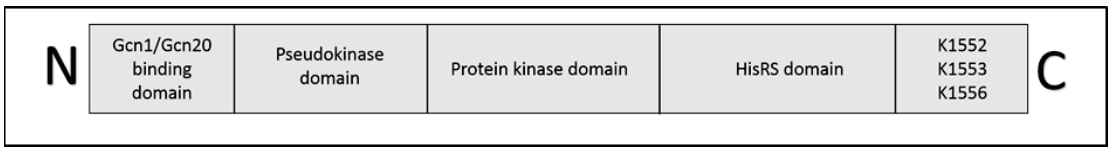


Figure 1.3 Schematic overview of the five characterised domains of Gcn2.

Gcn2 self-governs in part via auto-inhibitory mechanisms. The PK domain has been shown to interact with multiple domains of Gcn2. One of these interactions occurs between the PK and C-terminal domains, this association retains Gcn2 in an inhibitory conformation when Gcn2 activity is not required. A substitution mutation E803V of the PK domain weakens this auto inhibitory interaction and enhances the affinity of Gcn2 for tRNA, resulting in Gcn2 being constitutively active. The PK domain also associates with the flanking HisRS domain under non-starvation conditions, retaining Gcn2 in its latent state (J. Dong et al., 2000). Binding of $tRNA^{deacyl}$ to the Gcn2 HisRS domain disrupts the auto-inhibitory interaction between the HisRS and PK domains, and subsequent autophosphorylation of the threonine residues 882 and 887 in the PK activation loop enables full activation of Gcn2 (Qiu, Dong, Hu, Francklyn, & Hinnebusch, 2001b; Romano et al., 1998). In addition to the negative auto-inhibitory conformations formed between the Gcn2 domains, the PK domain also interacts with its N-terminal adjacent ψPK domain, this interaction however is reported to positively regulate latent Gcn2 activation upon binding of $tRNA^{deacyl}$ (Lageix, Rothenburg, Dever, & Hinnebusch, 2014).

1.1.6 Proteins that bind and regulate Gcn2

Gcn2 interacts *in vivo* with specific proteins which function in the regulation of its kinase activity. One of the most extensively characterised interactions occurs between Gcn2 and Gcn1. Gcn1 is a large protein that, like Gcn2, is conserved among eukaryotic organisms (Castilho et al., 2014). Gcn1 forms a complex with another regulatory protein, Gcn20 (M. Marton, D. Crouch, & A. Hinnebusch, 1993; Vazquez de Aldana, Marton, & Hinnebusch, 1995). Gcn2 cannot be activated in cells that have a deletion of *GCN1*, and cells with a *GCN20* deletion are not able to achieve full Gcn2 activation

when starved for amino acids (M. Marton et al., 1993; Vazquez de Aldana et al., 1995). In the absence of Gcn1, although Gcn2 cannot be activated upon amino acid starvation, Gcn2 is still capable of performing catalytic function (M. J. Marton, D. Crouch, & A. G. Hinnebusch, 1993). The Gcn1-Gcn20 complex is therefore believed to be essential for the transference of the starvation signal to Gcn2 but not essential for Gcn2 enzyme activity (Doerks, Copley, Schultz, Ponting, & Bork, 2002b; Donnelly, Gorman, Gupta, & Samali, 2013; M. Marton et al., 1993; Vazquez de Aldana et al., 1995).

The precise Gcn1 mediated mechanism for sensing starvation is yet to be confirmed, but there are two current models, both are initiated when accumulating $tRNA^{deacyl}$ binds to the ribosomal acceptor site (A site) (Grallert & Boye, 2007). The first model proposes that Gcn1 transfers the $tRNA^{deacyl}$ from the ribosomal A site to the Gcn2 HisRS domain. Alternatively, Gcn1 may serve as a scaffold protein that positions Gcn2 on the ribosome in a specific configuration that enables Gcn2 to transfer the $tRNA^{deacyl}$ from the ribosomal A site to its HisRS site (Marton, Vazquez de Aldana, Qiu, Chakraburtty, & Hinnebusch, 1997; Sattlegger & Hinnebusch, 2000b).In both models the $tRNA^{deacyl}$ must bind the Gcn2 HisRS domain for to Gcn2 to sense starvation and become activated.

Gcn2 is constitutively expressed and when amino acids are readily available for protein synthesis, it resides in the cell in latent form. During this time Gcn2 activity is repressed by intramolecular auto-inhibitory interactions. This facilitates a rapid Gcn2 mediated response upon amino acid starvation. (J. Dong et al., 2000; Qiu et al., 2001b). It is during the latent period that Gcn2 binds to eukaryotic translation elongation factor 1A (eEF1A).

eEF1A functions in the translation elongation cycle by delivering aminoacylated tRNA to the ribosomal A site (Taylor, Frank, & Kinzy, 2007). In addition to the cellular requirement for eEF1A activity during protein synthesis, under amino acid replete conditions, eEF1A was shown to bind to the C-terminus of the dormant Gcn2 *in vitro*. Dissociation of the eEF1A-Gcn2 interaction occurs when Gcn2 is bound by $tRNA^{deacyl}$ upon amino acid starvation (Visweswaraiah et al., 2011). Additionally eEF1A was shown to inhibit the phosphorylation of eIF2α by Gcn2, while exerting no influence on Gcn2 autophosphorylation, suggesting that eEF1A negatively regulates Gcn2 to inhibit

phosphorylation of eIF2α under amino acid-replete conditions (Visweswaraiah et al., 2011).

These observations led to the proposal of a mechanism by which eEF1A binds to Gcn2 to repress its activity under amino acid replete conditions. Upon amino acid starvation, the association is disrupted by the presence of $tRNA^{deacyl}$, which competes with eEF1A for binding to the Gcn2 C-terminus (Visweswaraiah et al., 2011).

1.1.7 The diverse functions of Gcn2

In addition to its canonical role in overcoming amino acid starvation, Gcn2 activity is necessary for the maintenance of cellular homeostasis during a diverse range of cellular stress conditions.

Gcn2 activation occurs in response to nutritional stresses such as glucose, purine or carbohydrate deprivation (Clemens, 1996; Pain & Clemens, 1991; Rolfes & Hinnebusch, 1993; Yang, Wek, & Wek, 2000). Additionally Gcn2 mediated stress responses have been reported under conditions of increased intracellular salinity or acidity (Goossens, Dever, Pascual-Ahuir, & Serrano, 2001; Hueso et al., 2012), and upon UV irradiation (Tvegard et al., 2007).

One of the processes in mammals that involves Gcn2 activity is the metabolism of lipids. Wild type mice with fed a leucine deficient diet were observed to utilise lipid stores and decrease fatty acid synthesis. Gcn2 deletion mice developed an opposing phenotype with an increase in fatty acid synthesis, decreased utilisation of lipid stores, abnormal accumulation of lipid in the liver as well as insulin resistance (Guo & Cavener, 2007).

In mammals Gcn2 also influences feeding behaviour, promoting a preference for amino acid balanced feed, over unbalanced feed (Maurin et al., 2005). Gcn2 has also been implicated in insulin sensitivity (Anthony et al., 2004), viral infection defence (Cosnefroy et al., 2013; J. Krishnamoorthy, Mounir, Raven, & Koromilas, 2008), immune response (H. Y. Liu et al., 2014; Ravindran et al., 2014) and tumour cell propagation (Wang et al., 2013; J. Ye et al., 2010).

As outlined above, Gcn2 is involved in a variety of significant processes required for organism homeostasis. For this reason Gcn2 has been implicated as a contributing factor in the development of a diverse range of diseases. The canonical role of Gcn2 in amino acid control reasonably associates the kinase with nutritional and metabolic disease, but there is overwhelming evidence that many more processes within an organism depend on the correct functioning of Gcn2.

Gcn2 activity contributes to the growth and maintenance of tumours, which means that it may be a viable target for cancer treatments. Tumour cells are often deprived of oxygen and nutrients due to insufficient blood supply and high metabolic demand. Tumour cell survival under conditions of amino acid or glucose scarcity is facilitated by activation of the integrated stress response pathway. *GCN2* deletion in mammals has been shown to impede tumour growth (Wang et al., 2013; Ye, Kumanova, Hart, Sloane, Zhang, De Panis, Bobrovnikova-Marjon, et al., 2010).

GCN2 mutations have been identified as the cause of pulmonary capilliary hemangiomatosis and pulmonary veno-occlusive disease in humans. These diseases are characterised by proliferation of the alveolar capillaries or septal veins/preseptal venules respectively, both are rare diseases with a poor prognosis (Best et al., 2014; Eyries et al., 2014).

The evidence of Gcn2 contribution in diseases such as these, infers that there is a requirement for the development of therapeutic agents that target Gcn2. Because Gcn2 is involved in such a wide range of cellular processes, it is first necessary to decipher the precise mechanisms of Gcn2 spatial and temporal regulation, to develop more targeted therapies. As it is likely that Gcn2 regulation is, in part, executed by Gcn2 binding proteins, these are promising targets for modulating specific Gcn2 function. However, evidence suggests that not all Gcn2 binding proteins are known yet (Castilho et al., 2014). Therefore, identification and characterisation of novel Gcn2 protein interaction partners will help to achieve a more comprehensive understanding of Gcn2 regulation.

1.2 Hypothesis and aim of this research

Gcn2 is an essential constituent of the GAAC pathway that enables organisms to endure periods of amino acid deficiency. Gcn2 activity has also been implicated in a vast array of additional cellular processes for the purpose of maintaining cellular homeostasis. Gcn2 regulation is multifaceted and includes inhibitory and facilitative protein interactions. Evidence strongly suggests that there are regulators which are yet to be identified (Castilho et al., 2014). Accordingly, large scale purification studies have identified many potential novel Gcn2 binding partners, although this data still needs to be critically analysed. Furthermore these studies did not capture all of the known Gcn2 binding proteins, suggesting that the experimental conditions were not optimal to identify the full repertoire of Gcn2 interaction partners. To gain a more complete understanding of the complex Gcn2 regulatory network, the identification and characterisation of novel Gcn2 protein interaction partners is necessary. I hypothesise that there are proteins yet to be discovered that bind to Gcn2 to positively or negatively regulate its activity.

In order to test this hypothesis, the aims of this study were as follows

- To critically evaluate published databases, to identify novel proteins that are likely in complex with Gcn2
- To screen candidate proteins to identify those that function in Gcn2 regulation
- To optimise a procedure that enables comprehensive identification of Gcn2 binding proteins by co-purification

Chapter 2

Materials and Methods

2.1 Biological Materials

The plasmids used in this study are listed in table 2.1 and the yeast strains are listed in table 2.2.

Table 2.1 *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source
BY4741	MATa *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0	Thermo Fisher
YDR283C	as BY4741 with *gcn2*Δ	Thermo Fisher
YLR096W	as BY4741 with *kin2*Δ	Thermo Fisher
YNL183C	as BY4741 with *npr1*Δ	Thermo Fisher
YGR097W	as BY4741 with *ask10*Δ	Thermo Fisher
YBL085W	as BY4741 with *boi1*Δ	Thermo Fisher
YLR342W	as BY4741 with *fks1*Δ	Thermo Fisher
YPR008W	as BY4741 with *haa1*Δ	Thermo Fisher
YHR158C	as BY4741 with *kel1*Δ	Thermo Fisher
YGR238C	as BY4741 with *kel2*Δ	Thermo Fisher
YGL197W	as BY4741 with *mds3*Δ	Thermo Fisher
YLR002C	as BY4741 with *noc3*Δ	Thermo Fisher
YDR477W	as BY4741 with *snf1*Δ	Thermo Fisher
YJR066W	as BY4741 with *tor1*Δ	Thermo Fisher
YPR115W	as BY4741 with *rgc1*Δ	Thermo Fisher
YER161C	as BY4741 with *spt2*Δ	Thermo Fisher
YER132C	as BY4741 with *pmd1*Δ	Thermo Fisher
YER167W	as BY4741 with *bck2*Δ	Thermo Fisher
YBL047C	as BY4741 with *ede1*Δ	Thermo Fisher
YNR051C	as BY4741 with *bre5*Δ	Thermo Fisher
YPL111W	as BY4741 with *car1*Δ	Thermo Fisher
YLR330W	as BY4741 with *chs5*Δ	Thermo Fisher
YML057W	as BY4741 with *cmp2*Δ	Thermo Fisher
YHR146W	as BY4741 with *crp1*Δ	Thermo Fisher
YGR187C	as BY4741 with *hgh1*Δ	Thermo Fisher
YMR109W	as BY4741 with *myo5*Δ	Thermo Fisher
YBL024W	as BY4741 with *ncl1*Δ	Thermo Fisher
YJR052W	as BY4741 with *rad7*Δ	Thermo Fisher
YDR465C	as BY4741 with *rmt2*Δ	Thermo Fisher
YLL002W	as BY4741 with *rtt109*Δ	Thermo Fisher
YDR181C	as BY4741 with *sas4*Δ	Thermo Fisher
YLL005C	as BY4741 with *spo75*Δ	Thermo Fisher
YOR009W	as BY4741 with *tir4*Δ	Thermo Fisher
YMR304W	as BY4741 with *ubp15*Δ	Thermo Fisher
YJR110W	as BY4741 with *ymr1*Δ	Thermo Fisher

YNL213C	as BY4741 with *rrg9*Δ	Thermo Fisher
YOL070C	as BY4741 with *nba1*Δ	Thermo Fisher
YMR255W	as BY4741 with *gfd1*Δ	Thermo Fisher
YDL182W	as BY4741 with *lys20*Δ	Thermo Fisher
YMR205C	as BY4741 with *pfk2*Δ	Thermo Fisher
YDR075W	as BY4741 with *pph3*Δ	Thermo Fisher
YNL201C	as BY4741 with *psy2*Δ	Thermo Fisher
YER173W	as BY4741 with *rad24*Δ	Thermo Fisher
YCR060W	as BY4741 with *tah1*Δ	Thermo Fisher
YHR155W	as BY4741 with *ysp1*Δ	Thermo Fisher
YBL058W	as BY4741 with *shp1*Δ	Thermo Fisher
YER151C	as BY4741 with *ubp3*Δ	Thermo Fisher
YKL101W	as BY4741 with *hsl1*Δ	Thermo Fisher
YPR052C	as BY4741 with *nhp6a*Δ	Thermo Fisher
YML010W	as BY4741 with *spt5*Δ	Thermo Fisher
YHR121W	as BY4741 with *lsm12*Δ	Thermo Fisher
YPR140W	as BY4741 with *taz1*Δ	Thermo Fisher
YGR192C	as BY4741 with *tdh3*Δ	Thermo Fisher
YLR150W	as BY4741 with *stm1*Δ	Thermo Fisher
YDR174W	as BY4741 with *hmo1*Δ	Thermo Fisher
YPL106C	as BY4741 with *sse1*Δ	Thermo Fisher
YLL024C	as BY4741 with *ssa2*Δ	Thermo Fisher
YAL005C	as BY4741 with *ssa1*Δ	Thermo Fisher
YHL034C	as BY4741 with *ssb1*Δ	Thermo Fisher
YNL025C	as BY4741 with *ssn8*Δ	Thermo Fisher
YMR116C	as BY4741 with *asc1*Δ	Thermo Fisher
YLR384C	as BY4741 with *iki3*Δ	Thermo Fisher
YKL143W	as BY4741 with *ltv1*Δ	Thermo Fisher

Table 2.2 Plasmids used in this study

Plasmid	Gene/s	Selectable marker	Vector	Source
pBY011	Vector	Amp, URA3	YCplac33	(Gietz & Sugino, 1988)
ScCD00096779	AAR2	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00094853	HRR25	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00010037	PRP31	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00095768	PRP4	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00097449	PRP6	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00096208	SNU114	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00097825	TIR4	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00097886	GFD1	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00096686	SAS4	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00094314	MDS3	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00011781	GIR2	Amp, URA3	pBY011	(Hu et al.,2007)
ScCD00010504	YIH1	Amp, URA3	pBY011	(Hu et al.,2007)
B3106	GCN2-FL-FLAG-6xHIS	URA3, 2µ	pEMBLyex	(Dong, Qiu et al. 2000)
B3107	GCN2-FL-Y1119L,R1120L	URA3, 2µ	pEMBLyex	(Dong, Qiu et al. 2000)
B3112	GCN2-FL-E803V	URA3, 2µ	pEMBLyex	(Dong, Qiu et al. 2000)
P1079	Vector	URA3, 2µ	pEMBLyex	(Dong, Qiu et al. 2000)
YGPM33d18 (16A6)	[YPL108W] ,PL107W, SSE1, YPL105C, MSD1, [FMP30]	Kan, LEU2, 2µ	pGP564	OPEN BIOSYSTEMS

YGPM32k14 (16B6)	[SSE1],YPL105C, MSD1, FMP30, [YPL102C], [ELP4]	Kan, LEU2, 2µ	pGP564	OPEN BIOSYSTEMS
YGPM8g08 (1B7)	[YBL029W], YBL028C, RPL19B, LSM2, RRN10, NCL1, [MCM2]	Kan, LEU2, 2µ	pGP564	OPEN BIOSYSTEMS
YGPM6o08 (1C7)	[NCL1], MCM2, [PIM1]	Kan, LEU2, 2µ	pGP564	OPEN BIOSYSTEMS
YGPM24b12 (11D11)	[MMS22], SFH1, VPS65, CWC24, PEX30, RPL38, YLR326W, YLR327C, tS(GCU)L, NMA1, REC102,[CHS5]	Kan, LEU2, 2µ	pGP564	OPEN BIOSYSTEMS
YGPM2m11 (11E11)	[NMA1], REC102, CHS5, JIP3, MID2, tD(GUC)L2, snR61, snR55, snR57, RPS25B, YLR334C, tE(UUC)L, NUP2, [SGD1]	Kan, LEU2, 2µ	pGP564	OPEN BIOSYSTEMS

2.2 Media

Yeast extract Peptone Dextrose (YPD)

Formedium Yeast extract	(1% w/v)
Formedium Peptone	(2% w/v)
Formedium Glucose	(2% w/v)

Yeast extract Peptone Glycerol (YPG)

Formedium Yeast extract	(1% w/v)
Formedium Peptone	(2% w/v)
Sigma-Aldrich Glycerol	(3% v/v)

Synthetic Dextrose (SD)

Formedium Yeast nitrogen base	(0.19% w/v)
Ajax Ammonium Sulfate	(0.5% w/v)
Formedium Glucose or Galactose	(2% w/v)

Luria-Bertini (LB)

Formedium Tryptone	(1% w/v)
Ajax Sodium Chloride	(0.5% w/v)
Formedium Yeast extract	(0.5% w/v)

pH 7

2.3 Media supplements

Amino acid solutions were prepared using MilliQ water as the solvent and autoclaved at 121°C and 15 psi for 20 minutes to sterilise. Amino acid solutions were stored at room temperature with the exception of histidine which is light sensitive and therefore stored at 4°C in a foil covered bottle. Amino acid supplements are listed in table 2.3. Antibiotics and starvation induction drugs were prepared using the specified solvent and are listed in table 2.4

Table 2.3 Amino acid media supplements

Amino Acid (formedium)	Final concentration mg/ml
Histidine	20.9
Isoleucine	6.6
Leucine	13
Methionine	7.5
Uracil	2.2
Valine	2.9

Table 2.4 Antibiotics and starvation induction drugs

Supplement	Solvent	Final concentration
Ampicillin (antibiotic)	Water	100 µg/ml
Sulfometuron methyl SM)	Dimethylsulfoxide	0.5-2 µg/ml
3-amino-Triazole (3AT)	Water	10 mM

2.4 Media Preparation

Liquid media

Liquid media for culture of yeast strains and bacteria was prepared using MilliQ filtered water as the solvent and autoclaved for sterilisation at 121°C and 15psi for 20 minutes. Media was then allowed to cool to room temperature before addition of sterile nutrient supplements. Media was stored at room temperature.

Solid media

Solid media was prepared using MilliQ water as the solvent, agar was added to the media at a final concentration of 2% (w/v) before autoclaving. The media was then cooled to 55°C and appropriate sterile nutrient supplements were added before being poured into petri dishes. The media solidified at room temperature overnight and was then stored at 4°C.

2.5 Growth conditions

Growth of *Saccharomyces cerevisiae*

Yeast cultures were streaked onto YPD, YPG or SD solid media and grown inverted at 30°C. Cultures were then stored on the solid media at 4°C.

To ensure sufficient access to oxygen, cultures grown in liquid media were incubated at 30 °C while shaking at 150 rpm.

Growth of *Escherichia coli*

Bacteria were streaked on LB media containing ampicillin and incubated inverted at 37°C. Cultures were stored at 4°C.

Liquid cultures were grown in LB media at 37°C while shaking at 150 rpm.

2.6 Long term storage of Yeast and bacterial strains

Yeast cultures were grown on solid media before inoculation into 30% (v/v) glycerol solution.

Bacterial cultures were grown in liquid media before inoculation into 70% (v/v) glycerol solution.

Yeast and bacterial cultures were then stored at -80°C.

2.7 Measuring optical density

The optical density of cultures was measured at 600nm using Bio Photometer plus (Eppendorf). The spectrophotometer was calibrated using distilled water and where appropriate the samples were diluted with water.

2.8 Semi quantitative growth assay

Appropriate strains were inoculated into 4ml SD media with required nutrient supplements and grown to saturation at 30°C. Four tenfold serial dilutions of each culture was prepared. 5µl of undiluted culture and each dilution was spotted onto the necessary solid media in conjunction with control strains. The agar plates were incubated at 30°C and growth was recorded for eight consecutive days using a document scanner.

2.9 Formaldehyde crosslinking

Yeast cultures were grown to saturation at 30°C and 150 rpm in 4ml of appropriate media. The optical density of each culture was determined and the appropriate volumes of SD with required nutritional supplements was inoculated to an optical density of 0.2. The cultures were grown at 30°C, 150 rpm until the necessary optical density was obtained. The cultures were then subjected to crosslinking for by transferring the culture to a centrifuge tube containing ice chips and formaldehyde to a final sample concentration of 1%. The samples were kept in ice and mixed by inversion every 15 minutes for 1 hour. The reaction was quenched by the addition of 2.5M Glycene to a

final concentration of 0.1M. The cells were then pelleted by centrifuging for at 4°C for 5 minutes at 4200 rpm.

2.10 Mechanical cell lysis for whole cell extract preparation

Yeast strains were grown to saturation at 30°C and 150 rpm in 4ml of appropriate media. The optical density of each culture was determined and appropriate volume of SD with required nutritional supplements was inoculated to an optical density of 0.2. The cultures were incubated at 30°C, 150 rpm until an optical density between 0.6 and 1 was obtained. The cultures were crosslinked using formaldehyde and amino acid starvation induction was performed by addition of SM to the culture to a final concentration of 1µg/ml for 1 hour where necessary. The cells were pelleted by centrifugation at a speed of 4200 rpm and a temperature of 4°C for 5 minutes. The pellets were frozen at -20°C overnight or for 1 hour at -80°. 1 pellet volume of acid washed glass beads and 200µl of cold breaking buffer was added to each sample and the cells were lysed using 10 cycles of vortexing for 30 seconds at high speed and standing on ice for 30 seconds. The cell lysate was separated from the glass beads by centrifugation at 4°C for 5 minutes at 4200 rpm and transferring the supernatant to a 1.7ml Eppendorf tube. The lysate was centrifuged for a further 10 minutes at 10,000 rpm to sediment the cell debris. The supernatant was extracted and the protein concentration was estimated using the Bradford protein assay method.

Breaking buffer I

30mM HEPES-KOH
50Mm KCl
Glycerol (10% v/v)
1mM PMSF
10µg/ml Pepstatin
1µg/ml Aprotinin
1µg/ml Leupeptin
5mM β-Mercaptoethanol

Breaking buffer II

30mM HEPES-KOH
50Mm KCl
Glycerol (10% v/v)
5mM β-Mercaptoethanol

Protease Inhibitor Cocktail tablet (Roche)
(1 tablet per 20ml buffer)

2.11 Bradford protein assay

Samples for analysis were diluted 100 Fold in a 96 well micro titre plate. Protein standards were also prepared containing Bovine Serum Albumin (BSA) concentrations that increase in 2µg increments from 2µg/µl to 14µg/µl. The total volume for all samples and standards was 50µl. 150µl of Prepared Bradford reagent was added to each of the samples and standards and their absorbance was measured at 595nm using a FLUOstar OPTIMA microplate reader.

2.12 Yeast whole cell extract preparation using chemical lysis

Yeast strains were grown to saturation at 30°C and 150 rpm in 4ml of appropriate media. The optical density of each culture was determined and 25ml of SD with required nutritional supplements was inoculated to an optical density of 0.2. The cultures were incubated at 30°C, 150 rpm until an optical density between 0.6 and 1 was obtained. The cultures were then subjected to crosslinking for 1 hour at 4° using a final concentration of 1% formaldehyde. Amino acid starvation induction was performed by addition of SM to the culture to a final concentration of 1µg/ml for 1 hour where appropriate. The cells were pelleted by centrifugation at a speed of 4200 rpm and a temperature of 4°C for 5 minutes. The cells were re-suspended in 1ml of sterile MilliQ water and 100µl was transferred to 0.6ml Eppendorf tubes and pelleted. The pellets were re-suspended in 200µl of 0.1M NaOH and left to incubate for 5 minutes at room temperature. The tubes were centrifuged at 4°C for 5 minutes at 4200 rpm and the supernatant was discarded.

2.13 Plasmid amplification and purification

5ml *Escherichia coli* cultures were grown overnight in LB media at 37°C shaking. A 2ml volume of the culture was centrifuged at 4200 rpm for 5 minutes. The cell pellet was re-suspended in 100µl of solution 1 with RNaseA to a final concentration of 0.2µg/µl. To lyse the cells 200µl of solution 2 was added, the sample was mixed by inversion and incubated at 20°c for 5 minutes before being placed on ice for 5 minutes. The reaction was neutralised by addition of 150µl of solution 3 to the sample, which sat for a further 5 on ice minutes before centrifugation at 10,000 rpm for 10minutes at 4°C

to pellet cell debris. The supernatant was transferred to a tube containing 450µl isopropanol and the plasmid DNA was pelleted by centrifuging at 20°C for a further 10 minutes at 10,000 rpm. The pellet was washed twice with 1 ml 70% ethanol and allowed to dry for 10 minutes before being suspended in 25µl of TE buffer.

Solutions used for *E.coli* plasmid purification

Solution 1	Solution 2	Solution 3
50 mM Glucose 10 mM EDTA 25 mM Tris-HCl (pH 8)	0.2 M NaOH SDS (1% w/v)	3 M KOac acetic acid (11% v/v)

TRIS EDTA (TE buffer) recipe
0.1M Tris-HCl (pH 8)
10 Mm EDTA

2.14 Agarose Gel Electrophoresis

A 1% (w/v) agarose gel was prepared in TAE buffer. 1µl of 10µg/µl stock solution of ethidium bromide per 100ml of TAE buffer was added to the gel before the gel was poured. The samples were prepared by mixing 1ul of sample to 4ul of loading buffer and loaded into the wells of the gel. The gel was run at 80V until the dye front had run approximately 70% of the gel length. An ultraviolet transilluminator was used to visualise the DNA bands and the images were captured and analysed using Quantity One software (BioRad).

50X Tris-Acetate EDTA (TAE)

2 M Tris
1 M Acetate
100 mM EDTA

DNA Loading Buffer (5X)

Sucrose(50% w/v)
0.1 mM EDTA
Xylene Cyanol (0.25% w/v)
Orange gelb (0.25% w/v)

2.15 Yeast transformation

Competent cell preparation

Yeast cultures were grown to saturation in 4ml YPD media at 30°C and 150 rpm. 49ml of YPD was then inoculated with 1ml of the saturated culture and incubated at 30°C and 150 rpm until an optical density of between 0.6 and 0.8 was reached. The samples were transferred to 50ml tubes and the cells were pelleted by centrifuging for 5 minutes at

4200 rpm and 4°C. The cell pellets were washed with 10ml of sterile MilliQ water, suspended in 500µl of solution I and incubated overnight at 4°C.

Yeast transformation

Herring sperm DNA was prepared by boiling at 65°C for 10 minutes and cooling in ice water. 300µl of solution II, 5µl of Herring sperm DNA and 5µl of plasmid DNA were mixed with 100µl of the competent yeast culture and incubated for 90 minutes at 150 rpm and 30°C. The cells were then heat shocked for 15 minutes at 43°C before being cooled in ice water for 5 minutes. The cells were pelleted by centrifuging for 3 minutes at 1000 rpm and the supernatant was discarded. The pellet was suspended in 100µl of SD medium, and the suspension was then dispersed on solid media using glass beads. The agar plates were incubated at 30°C for 2 to 4 days.

Solutions used for yeast transformation

Solution I	Solution II
10 mM Tris-HCl (pH 8)	10Mm Tris-HCl (pH 8)
1mM EDTA	1mM EDTA
100mM LiOAc	100mM LiOAc
	PEG (40% v/v)

2.16 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were prepared for gel electrophoresis by mixing quantities of sample and 2x protein loading dye and denaturing the samples at 85°C for 8 minutes. Two glass plates were cleaned, spacers were placed in between them and they were clamped together. The margins of the unit were sealed using a 7% agarose solution. The polyacrylamide gel was prepared by pipetting 20ml of 4% acrylamide pre-mix into one well of a gradient-mixer and 20ml of 17% acrylamide premix into the second well. 200µl of Ammonium Per Sulfate (APS 10% w/v) and 20µl of Tetramethylethylenediamine (TEMED) was added to each well and mixed. The acrylamide mixture was poured between the glass plates a comb was inserted into the upper opening and the gel was left at room temperature for 60 minutes to polymerise. After the gel had solidified the comb and lower spacers were removed, the wells were rinsed with distilled water and the gel was mounted to an electrophoresis unit. The prepared samples and a protein standard ladder (Invitrogen) were pipetted into the wells of the gel and electrophoresis was

performed at 250V and 100mA until the dye front had travelled between 7 and 10 centimetres.

4% acrylamide premix	75.3ml
MilliQ water	12ml
40% acrylamide (29:1)	30ml
Tris-HCl	1.2ml
SDS (10% w/v)	

17% acrylamide premix	48.8ml
MilliQ water	68ml
40% acrylamide (29:1)	40ml
Tris-HCl	1.6ml
SDS (10% w/v)	

Protein running buffer	100ml
10x Tris-Glycine	10ml
SDS (10% w/v)	890ml
MilliQ water	

Protein loading buffer	(0.1% w/v)
Bromophenol blue	(4% w/v)
SDS	(pH 6.8)
100mM Tris-HCl	(20% v/v)
Glycerol	(10% v/v)
β-Mercaptoethanol	

10x Tris-Glycene	
Tris Base	30.3 g/L
Glycine	144 g/L

2.17 Western blotting

The proteins that had been resolved in SDS-PAGE were transferred to 45µm (pore size) polyvinylidene difluoride membrane (Millipore). The membrane was hydrated for 5 minutes in methanol before equilibration using transfer buffer. The gel and membrane were arranged between layers of Whatman filter paper in a wet transfer unit and submerged in transfer buffer. The transfer was performed for 3 hours at 24V, 1A.

After the transfer was complete the membrane was rinsed with TBST and blocked for a minimum of 1 hour in a 5% (w/v) milk powder TBST solution. The membrane was washed for 10 minutes in TBST three times before incubation with the appropriate primary antibody for a minimum of 1 hour. The membrane was washed with TBST again three times for ten minutes and incubated with the appropriate secondary antibody for a minimum of 1 hour. The membrane was washed three times for ten minutes and sealed in a plastic bag before addition of enhanced chemiluminescence substrate. Visualisation of the chemiluminescence signal was performed using Fujifilm LAS-400 imaging system.

10X Tris Buffered Saline (TBS)

NaCl	80g/L
Tris base	24g/L
pH7	

Tris buffered Saline Tween (TBST)

10x TBS	(10% v/v)
Tween 20	(0.1% v/v)

Transfer buffer

Methanol	(20% v/v)
10x Tris-Glycene	(10% v/v)

Enhanced chemiluminescence substrate

Solution A

1M Tris (pH8.5)	
Luminol	4.4µg/ml
Coumaric Acid	0.6 µg/ml

Solution B

0.76M Tris (pH8.5)	
H_2O_2	(30% v/v)

Primary antibodies used in this study

Primary antibody	Dilution	Secondary antibody	Source
Gcn2	1:1000	Anti-guinea pig	Beatriz A Castilho (unpublished)
Gcn2	1:1000	Anti-Rabbit	Invitrogen
Gcn1	1:1000	Anti-Rabbit	Vasquez de Aldana et al 1995
Gcn20	1:1000	Anti-Rabbit	Vasquez de Aldana et al 1995
Pgk1	1:5000	Anti-mouse	Invitrogen
eIF2α-P	1:1000	Anti-Rabbit	Invitrogen

Secondary antibodies used in this study

Secondary antibody	dilution	Source
Anti-guinea pig	1:5000	Beatriz A Castilho (unpublished)
Anti-rabbit	1:100,000	Pierce
Anti-mouse	1:50,000	Pierce

Chapter 3

Identification of potential Gcn2 regulating proteins

3.1 Identification of putative Gcn2 binding proteins

Identification of *in vivo* protein interaction partners can provide valuable insight into the regulatory mechanisms and functions of a protein of interest. One of the methods utilised for the identification of these interaction partners is affinity purification. Affinity purification is a technique that takes advantage of the biochemical properties of an affinity tag to specifically bind to a ligand. Fusion proteins that feature a specific affinity tag are constructed, introduced into a host and expressed, this protein is therein referred to as the bait. A specific matrix capable of binding the affinity tag can then be used to capture the bait, enabling this protein to be effectively isolated along with any other interacting proteins that are bound to it (Figure 3.0), these bound proteins are referred to as prey (Li, 2010; Lichty, Malecki, Agnew, Michelson-Horowitz, & Tan, 2005; Rigaut et al., 1999). Once isolated, the precipitated complex can be subjected to Mass Spectrometry to identify the prey proteins that have co-purified in a complex with, or bound directly to, the bait protein of interest (Pottiez, 2015).

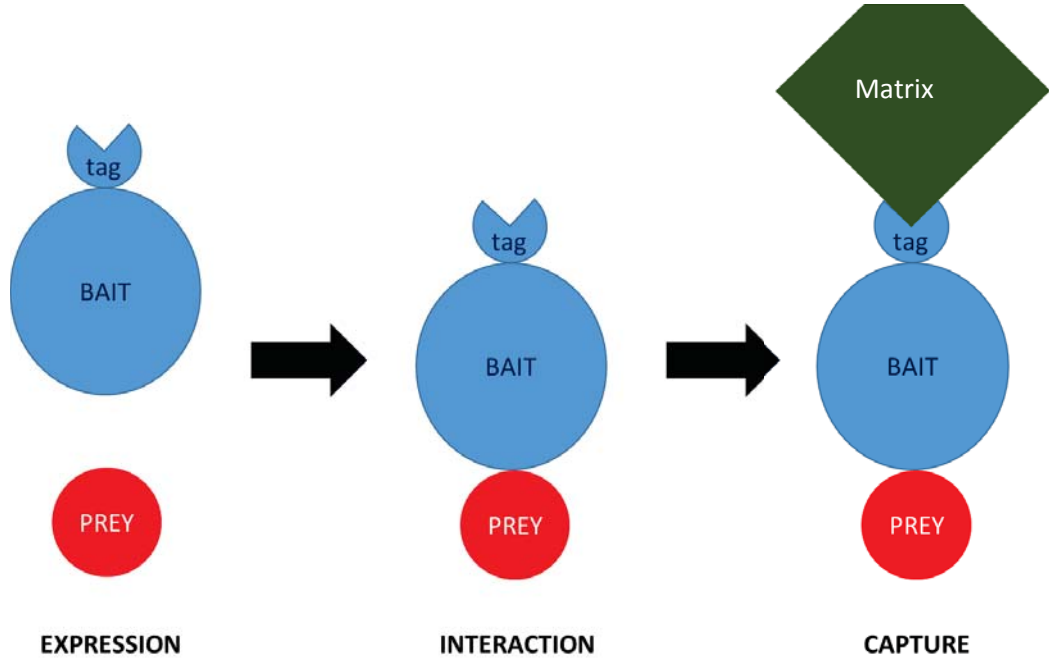


Figure 3.0 Schematic of Affinity Purification principle The tagged bait protein is expressed and binds to interacting prey proteins within the cell. A matrix capable of binding the affinity tag then captures the bait and bound prey proteins. The bait protein can then be purified and prey proteins that co-purified with the bait can be identified via Mass Spectrometry.

As the complete genome sequence of *Saccharomyces cerevisiae* is known (Goffeau et al., 1996), investigations into each individual gene can be performed by tagging the gene of interest with an epitope tag (Ghaemmaghami et al., 2003). Several independent research groups took advantage of this technique to perform large scale protein interaction studies. Data is currently available from five large scale high throughput affinity purification-mass spectrometry (AP-MS) publications. To identify proteins that potentially bind to Gcn2, or exist as part of a complex that includes Gcn2, we utilised the obtainable data from these five studies to construct the Gcn2 interactome. These large scale studies were performed by Ho et al 2002, Breitkreutz et al 2010, Krogan et al 2006, Gavin et al 2002 and Gavin et al 2006. All five studies used similar approaches to identify protein interactions using co-purification, however there were differences in the affinity tag used, bait protein expression levels, growth conditions of the yeast strains, methods of purification and prey protein identification.

Table 3.1 Comparison of large scale Affinity Purification Mass Spectrometry protein interaction studies

	Ho et al 2002	**Breitkreutz et al 2010**	**Krogan et al 2006**	**Gavin et al 2002**	**Gavin et al 2006**
Tap Epitope Used	C-terminal FLAG tag	C-terminal FLAG / HA	C-terminal TAP tag	C-terminal TAP tag	C-terminal TAP tag
Affinity Purification	one step	one step	two step	two step	two step
Bait Protein Expression	overexpress	Overexpress	native level	native level	native level
Detection Method	colloidal coomassie	western blot	silver staining	colloidal coomassie	colloidal coomassie
Trypsin Digestion	in gel	on bead	in gel	in gel	in gel
Mass Spectro-metry	LC-MS/MS	nLC-MS/MS	MALDI – TOF MS & LCMS/MS	MALDI – TOF MS	MALDI – TOF MS
Number of baits investigated	725	276	2357	1739	1993

In the study conducted by Ho et al in 2002 each gene coding for a bait protein was introduced into the host strain carried on a plasmid. The plasmid borne genes contained a C-terminal FLAG tag and transcription of the open reading frame (ORF) was regulated by a galactose inducible promotor. Because protein expression is regulated by this promotor, as opposed to the native promotor, expression of the proteins is

temporally controlled and the proteins are expressed at higher levels than are usually found in the cells. Single step affinity purification of the bait proteins was conducted using anti-FLAG resin and the proteins were then eluted from the beads using a competitive concentration of FLAG peptide suspended in HEPES-buffered saline (HBS). The samples were subject to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Colloidal coomassie staining was performed to visualise the purified protein complexes, followed by band excision and in gel trypsin digestion for protein extraction. The bait proteins and co-precipitated prey proteins were identified using Liquid chromatography Tandem mass spectrometry (LC-MS/MS) (Ho et al., 2002).

Similar to the Ho et al study, C-terminal tagged, plasmid borne ORFs under a galactose inducible promotor were used for bait protein expression in the study conducted by Breitkreutz et al in 2010. However in this study the fusion proteins were tagged with either FLAG or Human influenza hemagglutinin (HA) epitopes. Single step affinity purification was performed using anti-flag or anti-HA antibody bound to Protein A magnetic beads, followed by on bead protein trypsin digestion. Prey proteins were then identified using nano scale liquid chromatography tandem mass spectrometry (nLC-MS/MS) (Breitkreutz et al., 2010). This study focussed solely on protein-protein interactions involving protein kinases and phosphatases.

In the study conducted by Krogan et al 2006 and both studies conducted by Gavin et al (2002 and 2006), the C-terminal tandem affinity purification (TAP) tag was integrated into the chromosomal open reading frames (ORFs) via homologous recombination. This means that the bait proteins were expressed from their endogenous promotors at levels characteristic of the wild type strain. Affinity purification of the bait and its bound prey proteins was performed using a two-step process, consecutively exploiting the Protein A and calmodulin binding peptide domains featured in the TAP tag. First, the protein was captured using an immunoglobulin G antibody (IgG) matrix, which binds tightly to the Protein A domain of the TAP tag. A tobacco etch virus protease site located between the Protein A domain and the calmodulin binding peptide domain was then used to separate the bait from the Protein A domain in the TAP tag, thereby liberating the bait from the resin. The bait proteins were then recaptured via the calmodulin binding peptide (CBP) domain using Calmodulin-Sepharose beads, in the presence of Calcium. The bait protein and bound prey proteins were then eluted from the beads using

ethylene glycol tetra acetic acid (EGTA) which sequesters the calcium (Puig et al., 2001). In all three studies the eluate was then subjected to SDS-PAGE and the gel was stained to visualise proteins, the bands were excised and in gel trypsin digestion was performed followed by mass spectrometry. In the studies by both Krogan et al and Gavin et al Matrix-assisted laser desorption/ionization – time of flight Mass spectrometry (MALDI –TOF MS) was used to identify bait and prey proteins, however Krogan et al also utilised Liquid chromatography Mass Spectrometry (LCMS/MS) (A.-C. Gavin et al., 2002; A. C. Gavin et al., 2006; Krogan et al., 2006).

In summary the published studies each employed different methods for the identification of potential protein interactions (summarised in Table 3.1). The protein tag epitopes enabled purification of the bait protein using either a single step method (Figure 3.1) or alternatively a two-step procedure (Figure 3.2). FLAG and HA tag purification were conducted using single step affinity purification, this enables the process to be performed in a timely manner and reduces loss of prey proteins that may be bound to the bait by weak or transient interactions. Because the tagged protein is introduced into the cell using a plasmid vector and expression is governed by an inducible promotor, proteins that are natively expressed at low levels can be overexpressed with the introduction of the promotor stimulus. Overexpression of the protein strengthens weak and transient interactions due to the increased concentration of the bait protein driving interactions by mass action. However overexpression of a protein may also lead to interactions that normally don't occur in the cell. Therefore a potential consequence of this overexpression may be binding and subsequent MS identification of bait and prey interactions that occur only under these atypical circumstances (Chang, 2006; Ho et al., 2002). Tandem affinity purification using TAP tagged proteins eliminates potential complications that arise from overexpression of the bait protein, as the proteins are expressed at native levels from their endogenous promotor. The two stage purification process however is more time consuming and may result in the displacement of weakly interacting prey proteins from the bait, this means that the two-step process likely has decreased sensitivity compared to the single step purification. However the probability of non-specific protein contamination is also decreased due to the two-step purification process (Puig et al., 2001; Rigaut et al., 1999).

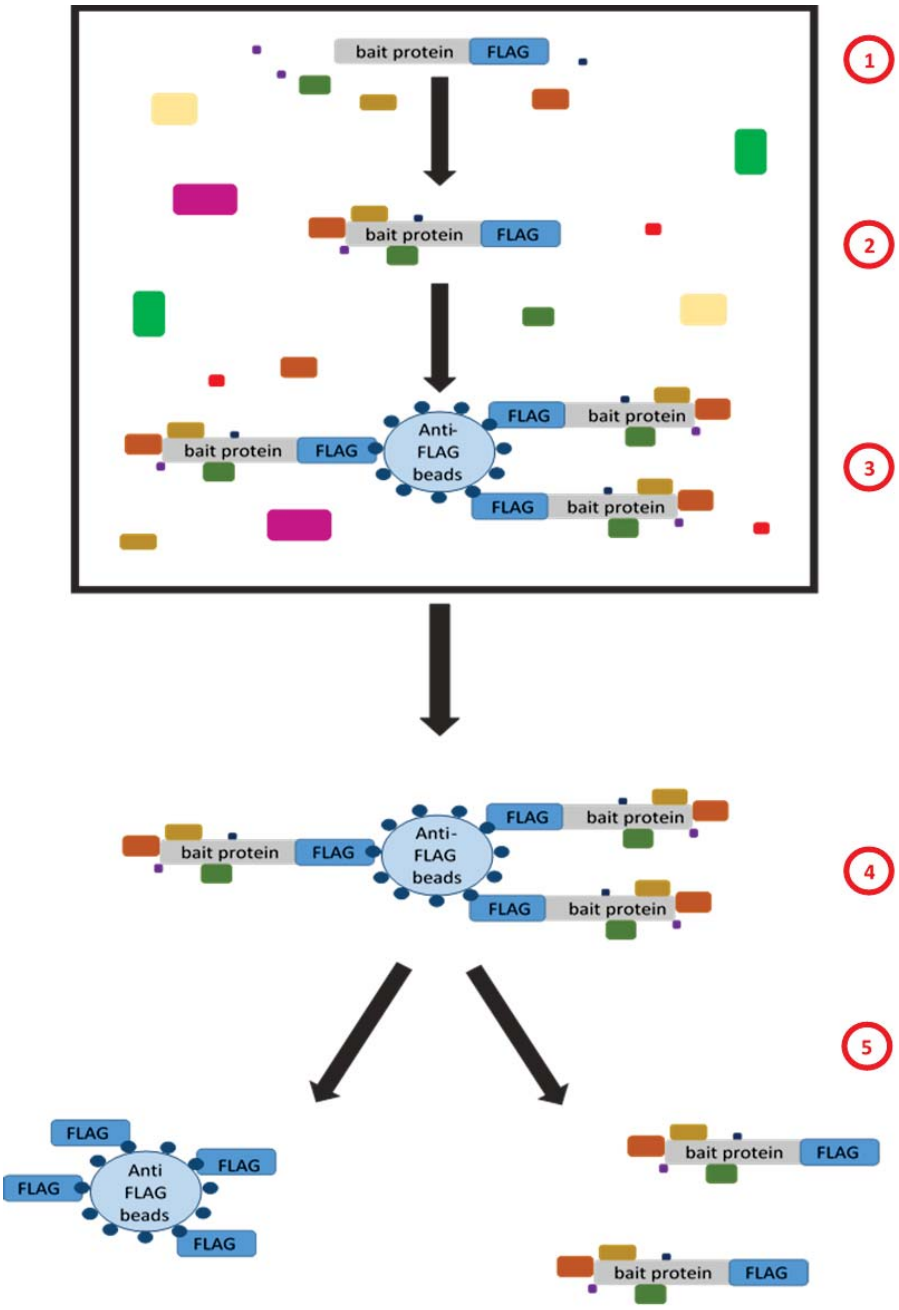


Figure 3.1 Overview of FLAG mediated affinity purification. 1) The recombinant bait protein is overexpressed from a galactose inducible promotor. 2) The bait protein binds to interacting proteins within the cell. 3) Anti-FLAG immobilised on a sepharose bead matrix captures the FLAG epitope of the recombinant protein. 4) The beads are washed to remove unbound proteins. 5) The bait and bound prey proteins are eluted from the beads using FLAG peptide suspended in HEPES-buffered saline (HBS). The principle is the same for HA tag affinity purification.

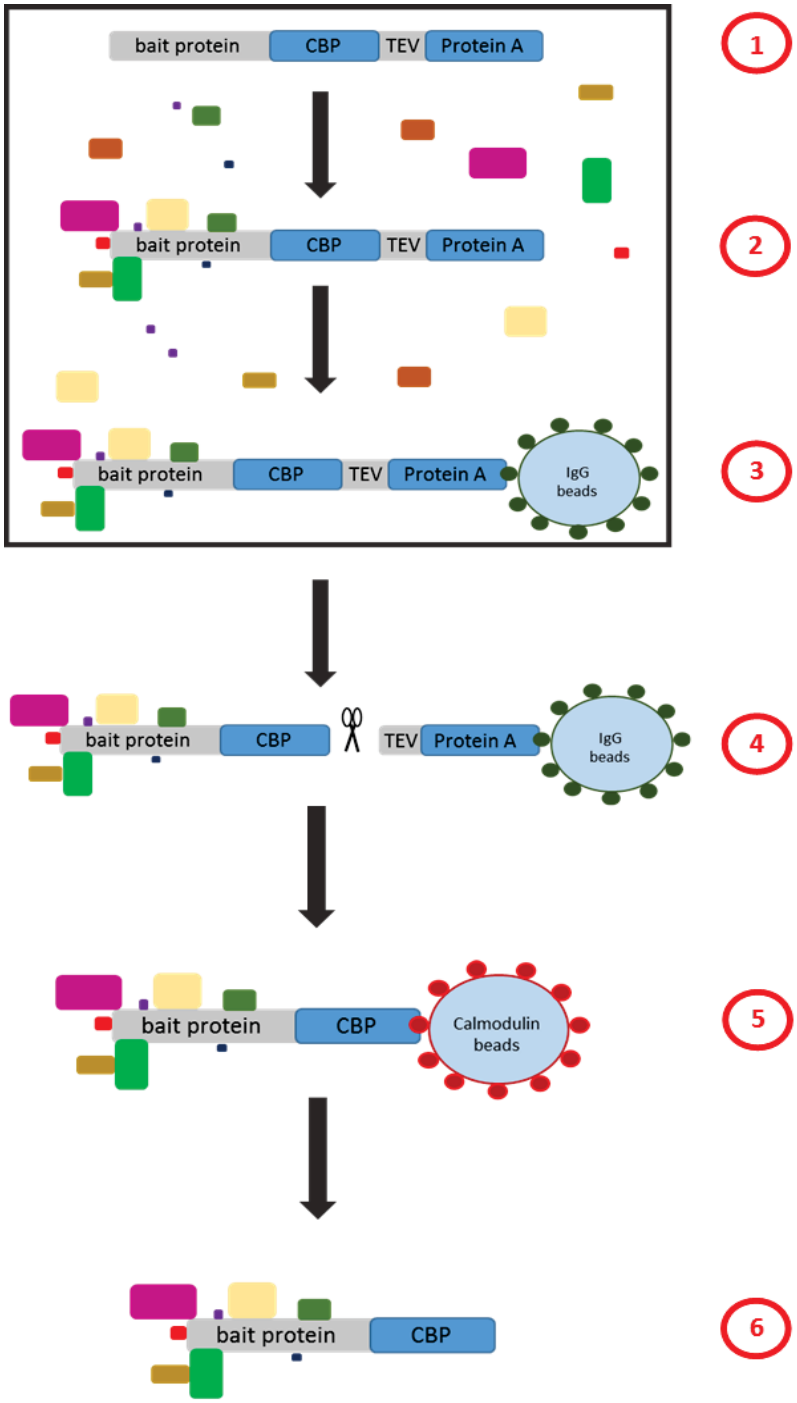


Figure 3.2 Overview of tandem affinity purification method using the TAP tag epitope. 1) The recombinant bait protein is expressed at native levels from its endogenous promotor. 2) The bait protein is bound by prey proteins. 3) The protein is captured using an IgG matrix which binds to a Protein A site of the TAP tag. 4) The Protein is cleaved from the IgG beads at a tobacco etch virus (TEV) protease site of the TAP tag. 5) The proteins are captured using calmodulin sepharose which binds the calmodulin binding peptide (CBP) of the TAP tag in the presence of Calcium 6) The bait protein and bound prey proteins are eluted from the beads using ethylene glycol tetra acetic acid (EGTA) which chelates the calcium ions.

The use of Gcn2 as a bait protein, and identification of Gcn2 as a prey protein in all of the five large scale affinity purification studies, as well as the use of different affinity purification epitope tags, facilitated our analysis of the published data. We reasoned that if a protein interaction was identified in more than one study, particularly if the studies utilised two separate epitopes, than there was an increased likelihood that it is a true interaction.

To construct the potential Gcn2 interactome, a dataset was compiled using the results from the five published large scale Affinity Purification studies. This raw Gcn2 interactome dataset included all proteins that co-precipitated with Gcn2 (bait) as prey, the bait proteins that co-precipitated Gcn2 as prey, and the prey proteins that were co-precipitated in parallel with Gcn2 (prey) by bait proteins (Figure 3.3). In total 1804 proteins were included in this compilation. It is likely that this raw Gcn2-interactome includes false positives, i.e. proteins that precipitated non-specifically bound to a Gcn2 containing complex or proteins that had unspecific affinity for the tag-binding matrix. Therefore this dataset was further analysed in the following chapters using two different approaches to identify proteins likely to be true members of a Gcn2-containing complex. These analysed datasets were called the primary and secondary Gcn2 interactomes (Figure 3.3).

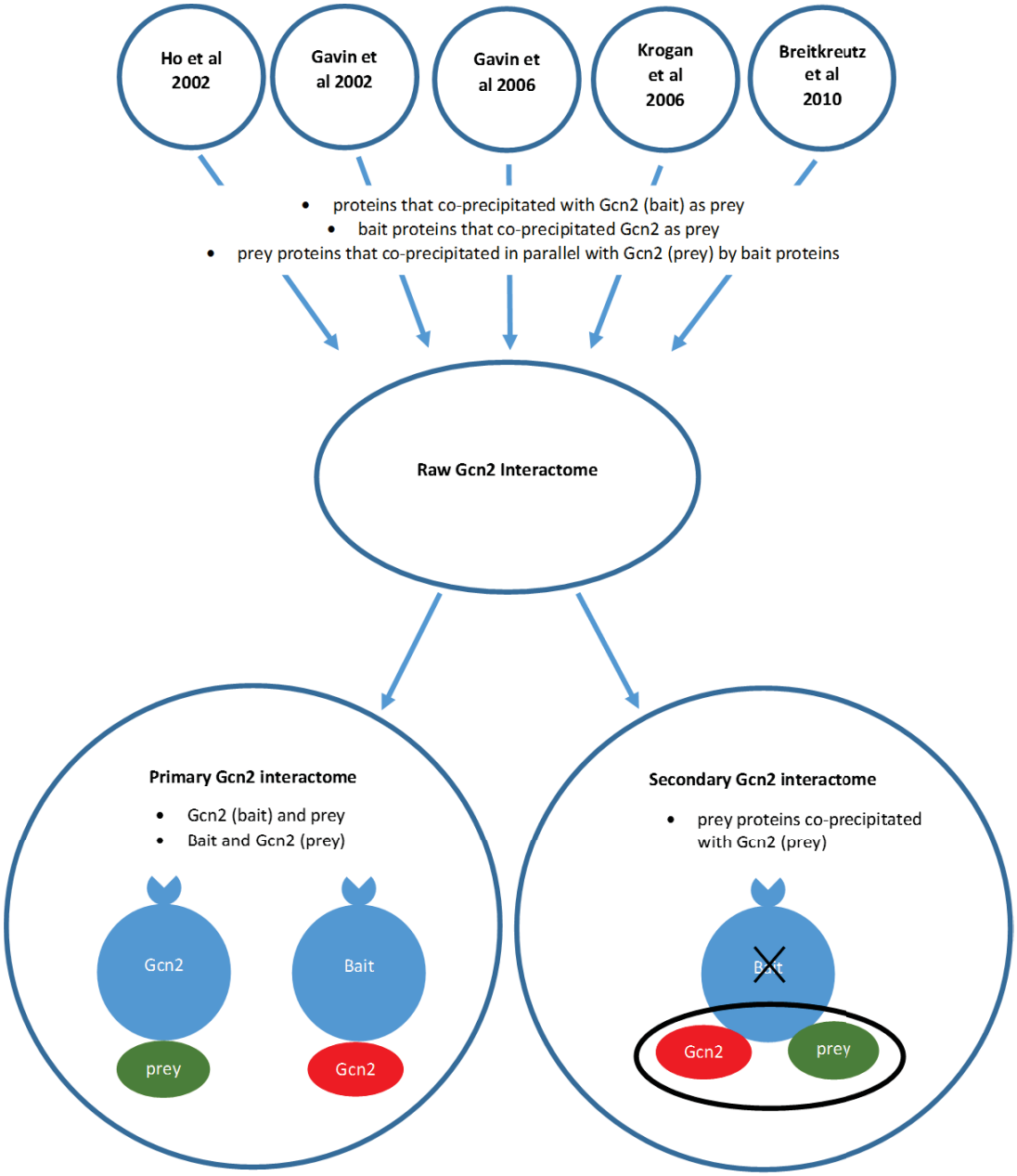


Figure 3.3 Schematic of Gcn2 interactome data analysis approach. The Gcn2 raw dataset was compiled using the results from the five published large scale Affinity Purification studies. This raw Gcn2 interactome dataset included all proteins that co-precipitated with Gcn2 (bait) as prey, the bait proteins that co-precipitated Gcn2 as prey, and the prey proteins that co-precipitated in parallel with Gcn2 (prey) by bait proteins. The Gcn2 raw dataset was further categorised into the primary Gcn2 and secondary Gcn2 interactomes. The primary Gcn2 interactome was comprised of all proteins that co-precipitated with Gcn2 (bait) as prey and bait proteins that co-precipitated Gcn2 as prey. The secondary interactome includes prey proteins that co-precipitated in parallel with Gcn2 (prey) by bait proteins in at least two studies.

3.1.1 Gcn2 Primary Interactome

Analysis of the compiled data was performed to identify proteins that were most likely to bind to, or form a complex with Gcn2 within the cell. This set of proteins will be referred to as the primary Gcn2 interactome.

The primary Gcn2 interactome was constructed by first filtering the raw Gcn2 interactome dataset to remove all of the proteins that did not co-precipitate Gcn2 as prey or were not co-precipitated as prey by Gcn2 (bait). The reasoning behind this was that if Gcn2 co-precipitates with a bait protein, or if Gcn2 co-precipitates a prey protein, then Gcn2 likely binds to, or is in a complex with that protein. However, if Gcn2 and another prey protein co-precipitate with the same bait protein, then Gcn2 is not necessarily in the same complex with the other co-precipitated prey protein. This is because we cannot exclude the possibility that the bait protein forms two distinct complexes, one containing Gcn2 and the other containing the other prey protein but not Gcn2. This filtering revealed 98 proteins potentially in complex with Gcn2. In 64 cases Gcn2 was used as bait, and in 32 cases Gcn2 was found as prey and in two cases Gcn2 was co-precipitated with a protein as both bait and prey.

The identified proteins may bind to Gcn2 directly, or they may be in a complex associating with Gcn2. For simplicity, in this thesis, all of these proteins will be referred to as Gcn2-binding proteins, even though they may bind to Gcn2 indirectly.

It was then reasoned that if Gcn2 was found to co-precipitate with one of the 98 candidate proteins in more than one of the five studies, than the interaction is most likely authentic. In particular if the two or more studies utilised different epitope tags in the purification procedure, as the different purification procedures are unlikely to co-precipitate the same contaminant proteins. Of the 98 Gcn2 candidate proteins there were eight proteins that were found to co-precipitate Gcn2 in two separate studies: Aar2, Prp31, Prp6, Prp4, Snu114, Snu66, Cdc48 and Hrr25. Two of these interactors, Cdc48 and Hrr25, were found in studies using different affinity purification epitope tags (Figure 3.4).

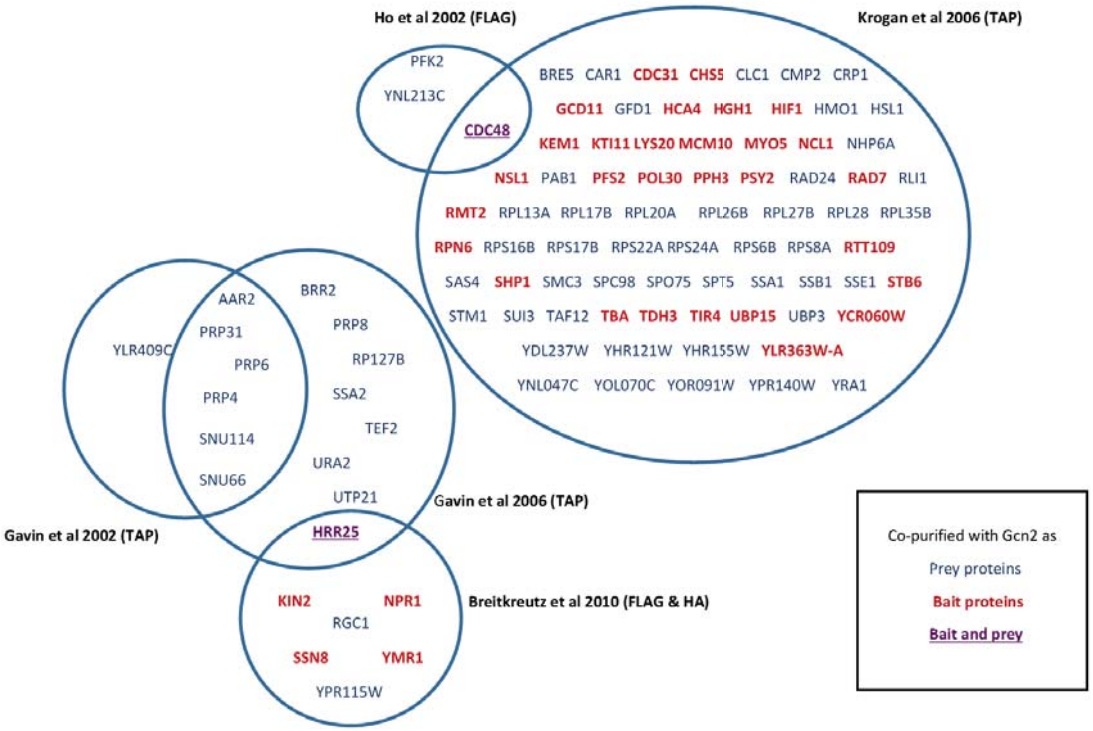


Figure 3.4 Gcn2 primary interactome. The primary interactome includes all proteins that bound to Gcn2 as bait or prey. Data was obtained from Ho et al (2002), Breikreutz et al (2010), Gavin et al (2002 and 2006) and Krogan et al (2006).

3.1.2 Gcn2 Secondary Interactome

In another approach, analysis of the data was performed on all prey proteins that were co-precipitated simultaneously with Gcn2 (prey) by a bait protein. The intention was to identify additional proteins that may form a complex that includes Gcn2. The reasoning was, that the more independent experiments that found a protein to co-precipitate along with Gcn2, the more likely it is in complex with Gcn2, in particular if this co-precipitation was observed with different bait proteins using different epitope tags.

All of the proteins that were featured in our raw Gcn2 interactome dataset were systematised by totalling the instances of occurrence of that protein within the dataset. The number of studies that identified each protein co-precipitating alongside Gcn2 as prey was then determined and all proteins that were shown to co-precipitate alongside Gcn2 in only a single study were excluded from this filtered dataset. The secondary interactome therefore features only proteins that have purified simultaneously with Gcn2 in more than one study, increasing the likelihood that these proteins form a complex that includes Gcn2.

Using these criteria, we found that out of the 1804 different proteins in the raw dataset, 43 of them co-precipitate with a bait protein that also simultaneously co-precipitated Gcn2 in at least 2 independent experiments. Indicating that these 43 proteins reside in a complex with Gcn2.

The proteins were further categorised into two sets, one in which the protein co-precipitated in parallel with Gcn2 by different baits carrying the same tag, the other set in which the protein co-precipitated alongside Gcn2 by different baits with different tags.

17 proteins were found in set 1, which co-precipitated in parallel with Gcn2 in two TAP tagged studies (Gavin et al 2006 and Krogan et al 2006). 26 proteins were found in the set 2, these co-precipitated alongside Gcn2 in one of the TAP tagged studies (Gavin et al 2006 or Krogan et al 2006) and also in the HA/FLAG tagged study (Breitkreutz et al 2010) (Figure 3.5). The proteins that co-precipitated alongside Gcn2 in two different studies using different epitope tags (TAP and HA) were deemed most likely to be in a complex that contains Gcn2 as the same contaminant proteins are less likely to be found using different purification procedures.

There were 6 proteins that featured in both the primary and secondary Gcn2 interactomes Rps17b, Rps22a, Ssa1, Ura2, Ypr115w and Hrr25, strongly suggesting that these proteins are Gcn2 interaction partners. These proteins may have co-precipitated directly bound to Gcn2, or indirectly bound through another protein present in a complex.

Next, additional analysis was performed which included proteins that were identified in both of the primary and secondary interactomes to further interpret the composition of Gcn2 complexes.

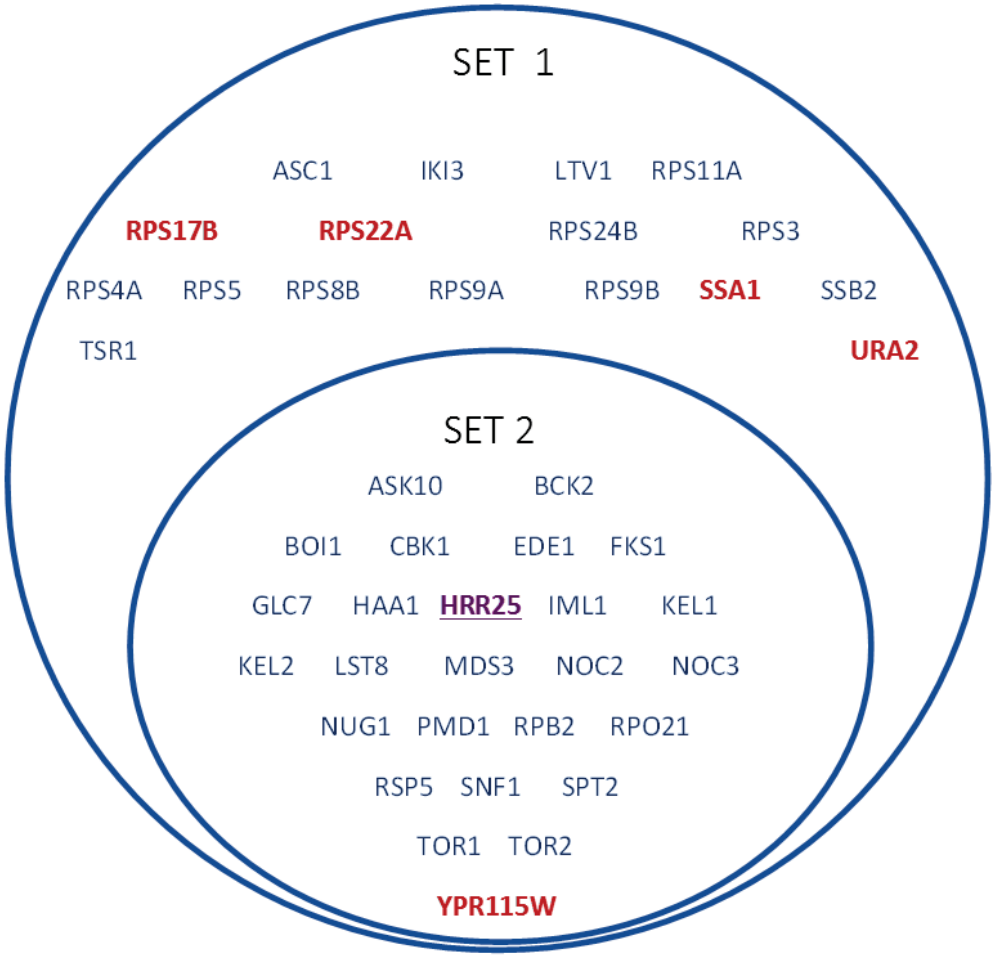


Figure 3.5 Gcn2 secondary interactome. Set 1) Proteins that were co-precipitated with Gcn2 by different baits in 2 TAP tagged studies Gavin et al 2006 and Krogan et al 2006. Set 2) Proteins that were co-precipitated with Gcn2 by different baits in one of the two TAP tagged studies Gavin et al 2006 and Krogan et al 2006 *and* the HA/FLAG tagged study Breitkreutz et al 2010. Proteins that also co-precipitated with Gcn2 (bait) are shown in red. HRR25 (purple and underlined) co-precipitated with Gcn2 as both bait and prey.

3.1.3 Identification of proteins that potentially form a complex with Gcn2

To further elucidate the protein composition of complexes that contain Gcn2, the data from the Gcn2 primary and secondary interactomes was examined with the intention of identifying groups of proteins that are consistently found together and with Gcn2. If the same proteins were found together in precipitation experiments irrespective of the bait used, this implies that the proteins may be part of the same complex. The analysis revealed three discernible potential complexes.

One of these putative complexes comprised Cdc48, Shp1 and Gcn2 (Figure 3.6). In the study conducted by Krogan et al (2006) both Shp1 and Gcn2 were identified as prey using TAP-tagged Cdc48 (bait) (Figure 3.6A). Correspondingly TAP-tagged Shp1 (bait) was found to co-precipitate Cdc48 and Gcn2 as prey proteins (Figure 3.6C), FLAG-tagged Gcn2 (bait) co-precipitated Cdc48 in the study conducted by Ho et al (2006) (Figure 3.6B), as indicated in the primary Gcn2 interactome. However Shp1 was not found to co-precipitate with Gcn2 in any of the affinity purification studies. It is possible that the purification conditions were too stringent leading to the loss of Shp1 during the experimental procedure. Nevertheless, these data suggest that Cdc28, Gcn2 and Shp1 are in the same complex. Supporting this idea, Cdc48 and Shp1 were reported previously to be in the same complex (Braun, Matuschewski, Rape, Thoms, & Jentsch, 2002; Schuberth, Richly, Rumpf, & Buchberger, 2004).

Our analysis now suggests that Gcn2 is part of this complex.

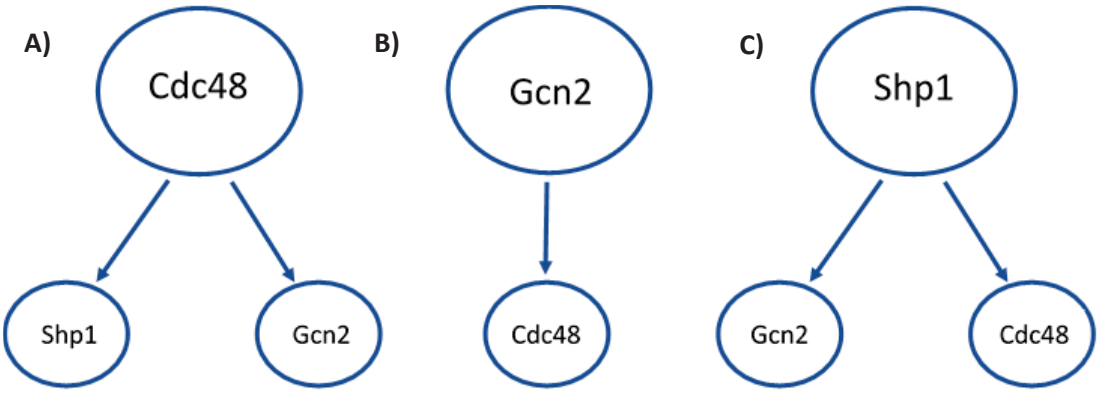


Figure 3.6 Identification of a potential protein complex that includes Gcn2, Cdc48 and Shp1. A) Cdc48 (bait) co-precipitated both Shp1 and Gcn2 (Krogan et al., 2006). **B)** Gcn2 (bait) co-precipitated Cdc48 (Ho et al., 2002). **C)** Shp1 (bait) co-precipitated Cdc48 and Gcn2 as prey (Krogan et al., 2006).

The second potential complex was composed of Gcn2 with three additional proteins, Gcd11, Tba1 and Hca4 (Figure 3.7). These three proteins all featured in the primary Gcn2 interactome as bait proteins, supporting the idea that each of these proteins are in complex with Gcn2. Gcn2 (bait) did not co-precipitate the proteins as prey in any of the five studies. This may be because these have dissociated during the purification procedures. Gcd11 (bait) co-precipitated Tba1 and Gcn2 as prey (Figure 3.7A). Tba1 (bait) co-precipitated Gdc11, Hca4 and Gcn2 as prey (Figure 3.7B). Gcn2 and Gcd11 (prey) were co-precipitated by Hca4 (bait) (Figure 3.7C) (Krogan et al., 2006). Collectively, these observations suggest that Gcd11, Tba1 and Hca4 are in the same complex with Gcn2.

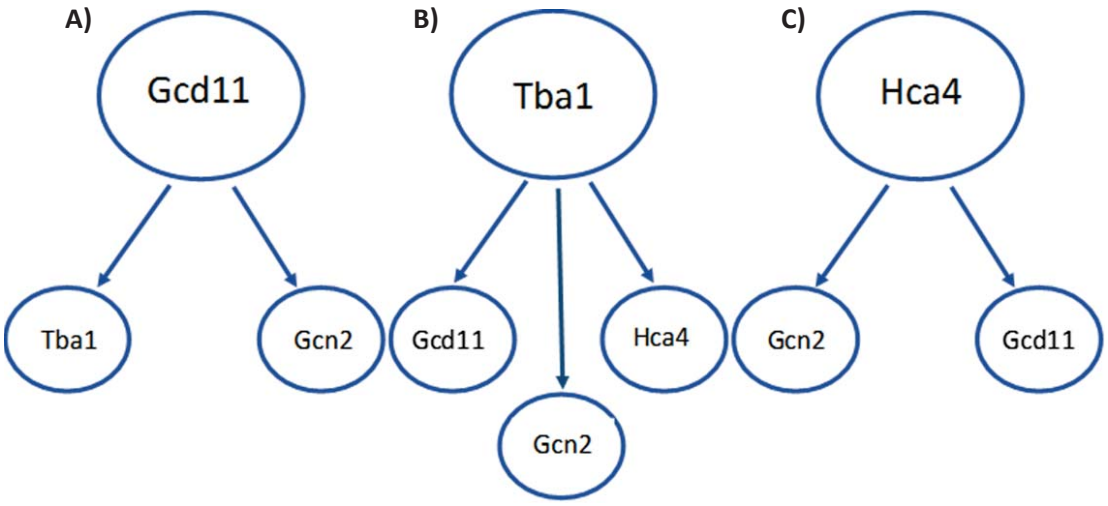


Figure 3.7 Identification of a potential protein complex that includes Gcn2, Tba1, Hca4 and Gdc11. A) Gcd11 (bait) co-precipitated Tba1 and Gcn2. **B)** Tba1 (bait) co-precipitated Gcd11, Gcn2, and Hca4**. C)** Hca4 (bait) co-precipitated Gcn2 and Gdc11 (Krogan et al., 2006).

The third potential complex comprised Gcn2, Pph3 and Psy2. Supporting this idea, it has been previously determined that Pph3 and Psy2 do form a complex (Bryan M. O'Neill et al., 2007). Pph3 and Psy2 (bait) each co-precipitated Gcn2 and the third protein (Krogan et al., 2006) (Figure 3.8). Both bait proteins co-precipitating Gcn2 alongside the other protein as prey indicated that the two proteins may form a complex which includes Gcn2.

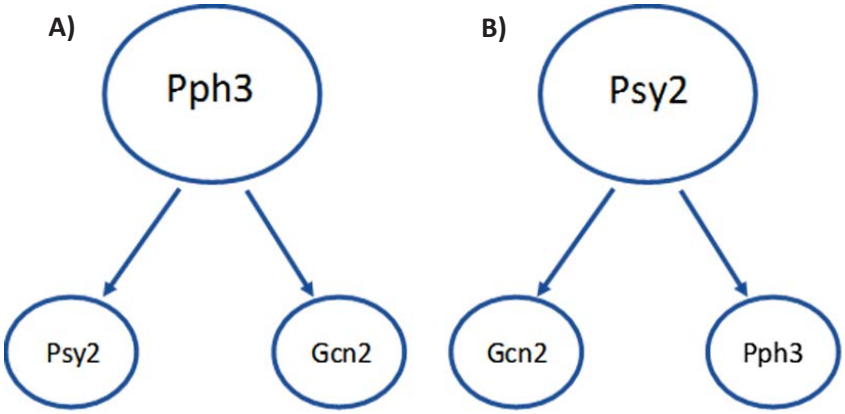


Figure 3.8 Identification of a potential protein complex that includes Gcn2, Pph3 and Psy2. A) Pph3 (bait) co-precipitated Psy2 and Gcn2. **B)** Psy2 (bait) co-precipitated pph3 and Gcn2 (Krogan et al., 2006).

3.2 Identification of potential Gcn2 regulating proteins

The interactome analysis identified potential Gcn2 binding proteins. To attempt to validate all of these proteins as true binding partners of Gcn2 would be extremely time consuming, we decided to instead screen for the Gcn2-binding proteins that affect Gcn2 activity. Specifically, we aimed to identify the proteins that are required for the activation of Gcn2.

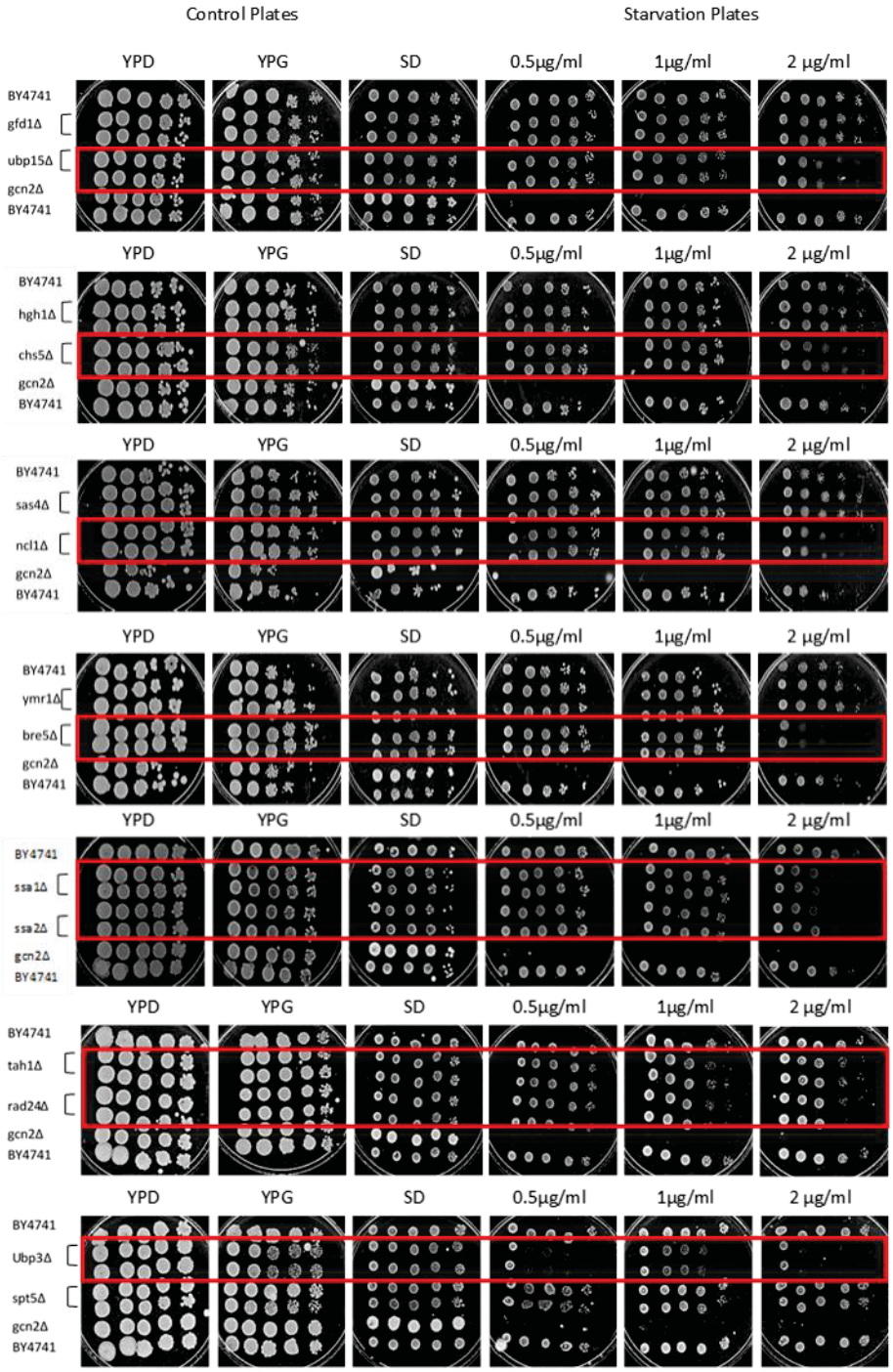
Gcn2 is activated by starvation for even a single amino acid (J. Dong et al., 2000). Because activation of Gcn2 enables cells to overcome inadequate amino acid supply, growth assays can be conducted to assess potential Gcn2 activation using specific agents that induce amino acid starvation. Strains that grow on media at a rate comparable to that of the wild type under amino acid limitation can be presumed to have sufficient Gcn2 activation. Strains with impaired Gcn2 function are unable to overcome starvation, and therefore display impaired growth under amino acid starvation conditions. With this semi-quantitative growth assay, Gcn2 activity can be easily scored in a semi-quantitative manner in living cells.

3.2.1 Screen of single gene deletion mutants for sensitivity to sulfometuron methyl

In order to test whether a protein is required for Gcn2 activity or activation, we performed semi-quantitative growth assays using yeast strains that have a deletion of the corresponding gene. For that we took advantage of the commercially available yeast library containing yeast strains each with a single deleted gene (Thermo Fisher). Deletion of essential genes however renders cells inviable, therefore it could not be tested whether the corresponding proteins are required for Gcn2 activation. Proteins that were identified in the interactome which had been previously characterised were excluded from the screen, these include Gcd11 (eIF2γ) (Harashima & Hinnebusch, 1986a), Sui3 (eIF2β) (Donahue, Cigan, Pabich, & Castilho Valavicius), Tef2 (eEF1A)(Visweswaraiah et al., 2011), Kem1, Clc1 (Shanmugam and Sattlegger unpublished) and all ribosomal proteins (Jochmann and Sattlegger unpublished).

Of the 136 proteins that featured in the primary and secondary Gcn2 interactomes, 61 viable deletion strains were available. One of the strains however failed to proliferate under optimal growth conditions, therefore a total of 60 strains were screened.

Cultures of the wild type and gcn2 deletion control strains and the viable gene deletion mutants were grown to saturation, tenfold serial dilutions were performed and then 5µl of each dilution was spotted onto solid media. Yeast peptone dextrose (YPD) and Synthetic dextrose (SD) media were used for growth rate evaluation of the deletion strains in reference to the isogenic wild type under replete conditions. YPD is a complete medium containing yeast extract, allowing for maximum growth rate. SD medium is minimal medium which served a direct reference for the SD medium containing the amino acid starvation inducing drug. Yeast peptone glycerol (YPG) media contains equivalent nutrient composition to YPD, however glycerol is supplied as the carbon source. YPG can therefore be used to identify "petites" which are unable to thrive on non-fermentable carbon sources due to mitochondrial defects (Ferguson & Von Borstel, 1992). Three starvation plates were also used in each assay, these plates contain SD media each with a different concentration of sulfometuron methyl (SM), 0.5µg/ml, 1 µg/ml and 2 µg/ml. SM is an agent that induces starvation for the branched chain amino acids by inhibition of the enzyme Acetolactate synthase (ALS) which catalyses an essential step in the biosynthesis of Leucine, Isoleucine and Valine (Falco & Dumas, 1985; LaRossa & Schloss, 1984). Three control strains were used in each assay. Two cultured single colony strains of BY4741, which is the wild type strain isogenic to the yeast deletion strains, were used as a positive control for growth under starvation conditions. An isogenic *gcn2Δ* was used a negative control. Since Gcn2 is deleted from this strain no growth of this strain occurs when starved for amino acids. Strains that were observed to have disrupted growth in the presence of SM were referred to therein as SM sensitive or SM^s. The growth assay was conducted at 30°C and the growth was documented daily using a document scanner.


Control Plates
Starvation Plates
YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
gfd1Δ
ubp15Δ
gcn2Δ
BY4741
hgh1Δ
chs5Δ
sas4Δ
ncl1Δ
ymr1Δ
bre5Δ
ssa1Δ
ssa2Δ
tah1Δ
rad24Δ
Ubp3Δ
spt5Δ

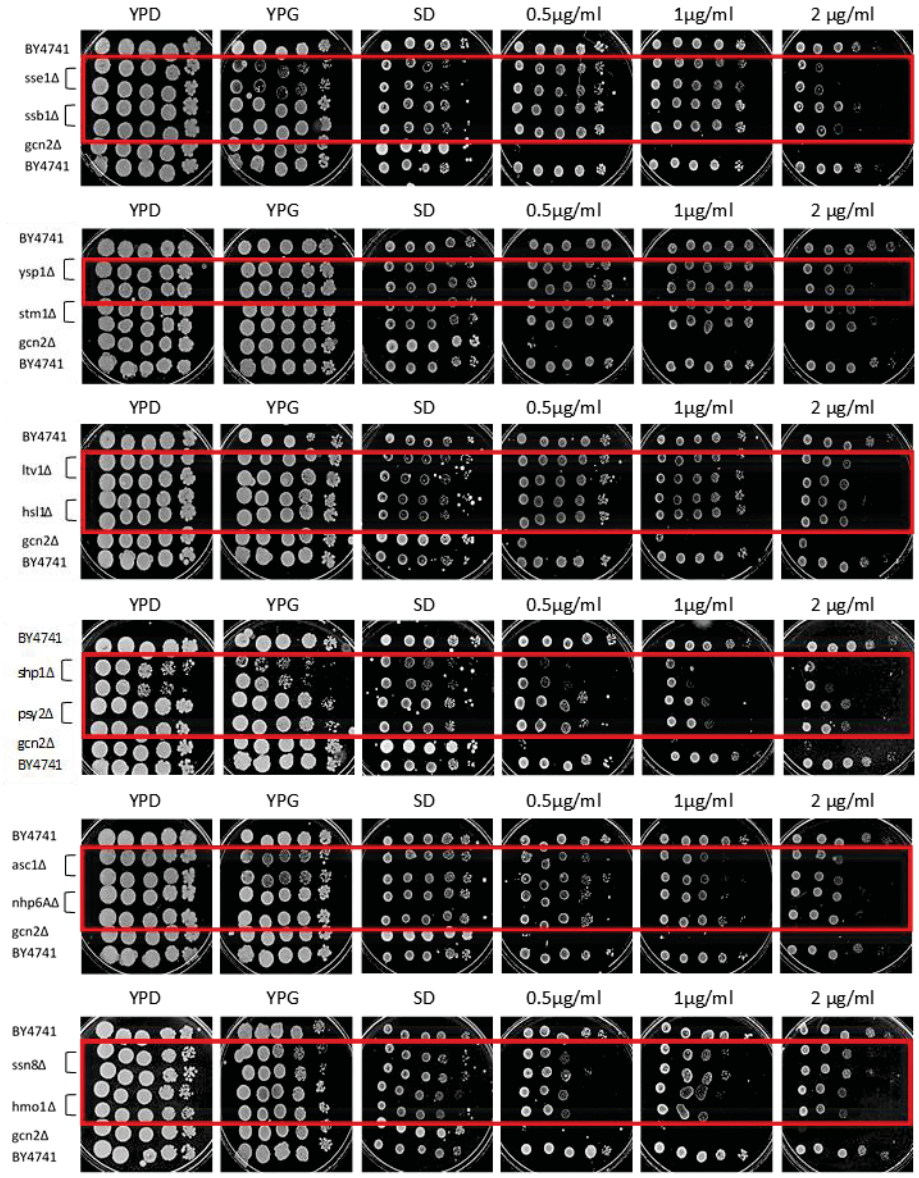


Figure 3.9 Single gene deletion mutants that displayed sensitivity to sulfometuron methyl. Cultures of the indicated strains were grown to saturation in liquid media. 10 fold serial dilutions of the cultures was performed and 5µl of each dilution was spotted onto the indicated agar plates. YPD, YPG, SD and SD containing SM at the specified concentration (0.5µg/ml, 1 µg/ml or 2 µg/ml). The plates were then incubated at 30°C and scanned daily for 7- 10 days using a document scanner to record cell growth. For results of entire screen see appendix 1.

As anticipated the wild type strain BY4741 thrived on all types of media in all of the semi quantitative growth assays performed and the *gcn2Δ* strain grew only on media that did not contain SM. There were two strains that exhibited a petite phenotype, *snf1Δ* and *rrg9Δ*, as it was not possible to score them for SM^s they were therefore excluded from subsequent analysis. Of the 60 mutant strains assayed, 20 were found to display SM sensitivity to varying degrees (Figure 3.9).

In order to gauge the degree of SM sensitivity displayed by each strain, the growth of each dilution was quantified on the SD control plate and each of the three SM containing plates. Each dilution was assigned a score based on a visual estimation of the growth of the dilution spot. When a spot reached full growth the score was 2 points, if growth could be well seen but it was not to the full possible extent it was scored 1 point and weak growth was scored 0.5 (appendix 2 growth scores of each strain). For each strain a total score was assigned by adding the scores of all dilutions. In order to take any growth defects in the absence of SM into consideration, a growth ratio was obtained by dividing the final score of a strains on each concentration of SM by that of the control plate. In order to relate the mutant strain's growth to that of the wild-type, this growth ratio was then divided by the ratio of the wild type (BY4741). This score was called the final growth score of each strain, for each concentration of SM. A score of 1 would therefore indicate a growth of the strain equivalent to that of the wild type.

There were two strains, *ubp3Δ* and *shp1Δ*, which were observed as having a strong SM sensitivity with a score lower than 0.5 for all three concentrations of SM. Eight strains showed moderate SM sensitivity with a score lower than 0.8 for at least two out of three concentrations of SM: *ysp1Δ*, *psy2Δ*, *asc1Δ*, *nhp6aΔ*, *ssn8Δ*, *hmo1Δ*, *tah1Δ* and *rad24Δ*. A further ten strains displayed weak SM sensitivity with a score lower than 0.8 apparent only when grown on the media containing the highest concentration of SM (2µg/ml): l*tv1Δ, hsl1Δ ssa1Δ, ssa2Δ, sse1Δ, ssb1Δ, bre5Δ, ncl1Δ, chs5Δ* and *ubp15Δ* (Table 3.2).

Table 3.2 Final SM sensitivity scores for all deletion strains subjected to semi-quantitative growth assays

Strain	SM (0.5µg/ml)	SM (1µg/ml)	SM (0.5µg/ml)	SM sensitivity
kin2Δ	1.00	1.00	1.00	
npr1Δ	1.00	1.00	1.00	
ask10Δ	1.00	1.00	1.00	
fks1Δ	1.00	1.00	1.00	
boi1Δ	1.00	1.00	1.00	
haa1Δ	1.00	1.00	1.00	
kel1Δ	1.07	1.00	1.00	
kel2Δ	1.07	1.00	1.00	
mds3Δ	1.06	1.00	1.00	
snf1Δ	1.06	1.06	1.06	
tor1Δ	1.00	1.00	1.00	
rgc1Δ	1.00	1.00	1.00	
spo75Δ	1.00	1.00	1.00	
rtt109Δ	1.00	1.00	1.00	
cmp2Δ	1.00	1.00	1.00	
pfk2Δ	1.00	1.00	1.00	
gfd1Δ	1.00	1.00	1.00	
ubp15Δ	1.00	1.00	.733	Weak SM^s
tir4Δ	1.00	1.00	1.00	
nba1Δ	1.00	1.00	1.00	
car1Δ	.999	.999	.999	
pph3Δ	.999	.999	.999	
hgh1Δ	.999	.999	.999	
chs5Δ	1.07	1.07	.782	Weak SM^s
sas4Δ	1.00	1.00	1.00	
ncl1Δ	1.00	1.00	.5	Weak SM^s
rrg9Δ	1.07	1.00	1.00	
myo5Δ	1.07	1.00	1.00	
ymr1Δ	1.00	1.00	1.00	
bre5Δ	1.00	1.00	.26	Weak SM^s
rad7Δ	1.00	1.00	1.00	
lys20Δ	1.00	1.00	1.00	
crp1Δ	1.00	1.00	1.00	
rmt2Δ	1.00	1.00	1.00	
bck2Δ	1.00	1.00	1.06	
ede1Δ	1.00	1.00	1.06	
spt2Δ	1.00	1.00	.944	
pmd1Δ	1.00	1.00	1.00	
ssa1Δ	1.06	1.00	.454	Weak SM^s
ssa2Δ	1.06	1.06	.756	Weak SM^s
sse1Δ	.106	.941	.353	Weak SM^s
ssb1Δ	1.13	.800	.750	Weak SM^s
tdh3Δ	1.20	1.20	1.00	

Table 3.2 continued final SM sensitivity scores for all deletion strains subjected to semi-quantitative growth assays

lsm1Δ	1.20	1.20	1.00	
ysp1Δ	.900	.757	.667	SM^s
stm1Δ	1.08	1.08	.933	
ltv1Δ	.941	.941	.764	Weak SM^s
hsl1Δ	.941	.941	.764	Weak SM^s
iki3Δ	1.28	1.28	1.28	
taz1Δ	1.28	1.28	1.21	
shp1Δ	.429	.322	.322	Strong SM^s
psy2Δ	.875	.633	.633	SM^s
asc1Δ	.555	.555	.626	SM^s
nhp6aΔ	.611	.666	.688	SM^s
ssn8Δ	.588	.588	.588	SM^s
hmo1Δ	.600	.600	.533	SM^s
tah1Δ	.8125	.750	.750	SM^s
rad24Δ	.882	.882	.764	SM^s
ubp3Δ	.300	.450	.222	Strong SM^s
spt5Δ	1.00	1.00	1.00	

3.2.2 Screening SM^s strains for those with impaired Gcn2 function

In total there were 20 mutants that displayed a SM^s phenotype. SM sensitivity was used as an indicator of impaired Gcn2 function, however sensitivity to SM may be due to an unrelated effect such as enhanced import of the drug into the cells. It therefore needed to be validated whether the observed SM^s phenotype was due to impaired Gcn2 activation. The direct consequence of Gcn2 activation is the phosphorylation of eIF2α (S. A. Wek, S. Zhu, & R. C. Wek, 1995b). It is possible to determine whether the SM sensitivity observed in the gene deletion mutants results from an inability to activate Gcn2 by measuring the levels of phosphorylated eIF2α (eIF2α-P) within the cell. Cells that cannot activate Gcn2 under amino acid limiting conditions will have lower quantities of eIF2α-P when subjected to SM starvation compared to the starved wild type strain.

To test the eIF2α-P levels of the mutant strains, two identical cultures of each strain were grown to exponential phase. One culture was used to determine eIF2α-P levels under replete conditions and the other under amino acid starvation conditions. In the latter, starvation was induced by the addition of SM to a final concentration of 1μg/ml for 1 hour at 30°C. Both sets of each strain were crosslinked by addition of 1% formaldehyde. Formaldehyde is an extremely small molecule that can pass through cell walls and membranes rapidly (Orlando, Strutt, & Paro, 1997). Once in the cell, the formaldehyde induces the formation of covalent bonds between amino acid side chains, instantaneously stopping any enzymatic reactions and preserving the molecular interactions (Klockenbusch, O'Hara, & Kast, 2012; Orlando et al., 1997). Formaldehyde crosslinking was performed for 1 hour at 4°C after which the remaining unreacted formaldehyde was quenched using glycine at a final concentration of 0.1M. Cell extracts were prepared and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. eIF2α-P was detected with the use of a specific antibody that detects only the phosphorylated form of eIF2α Phosphoglycerate kinase (Pgk1) was also detected as a loading control.

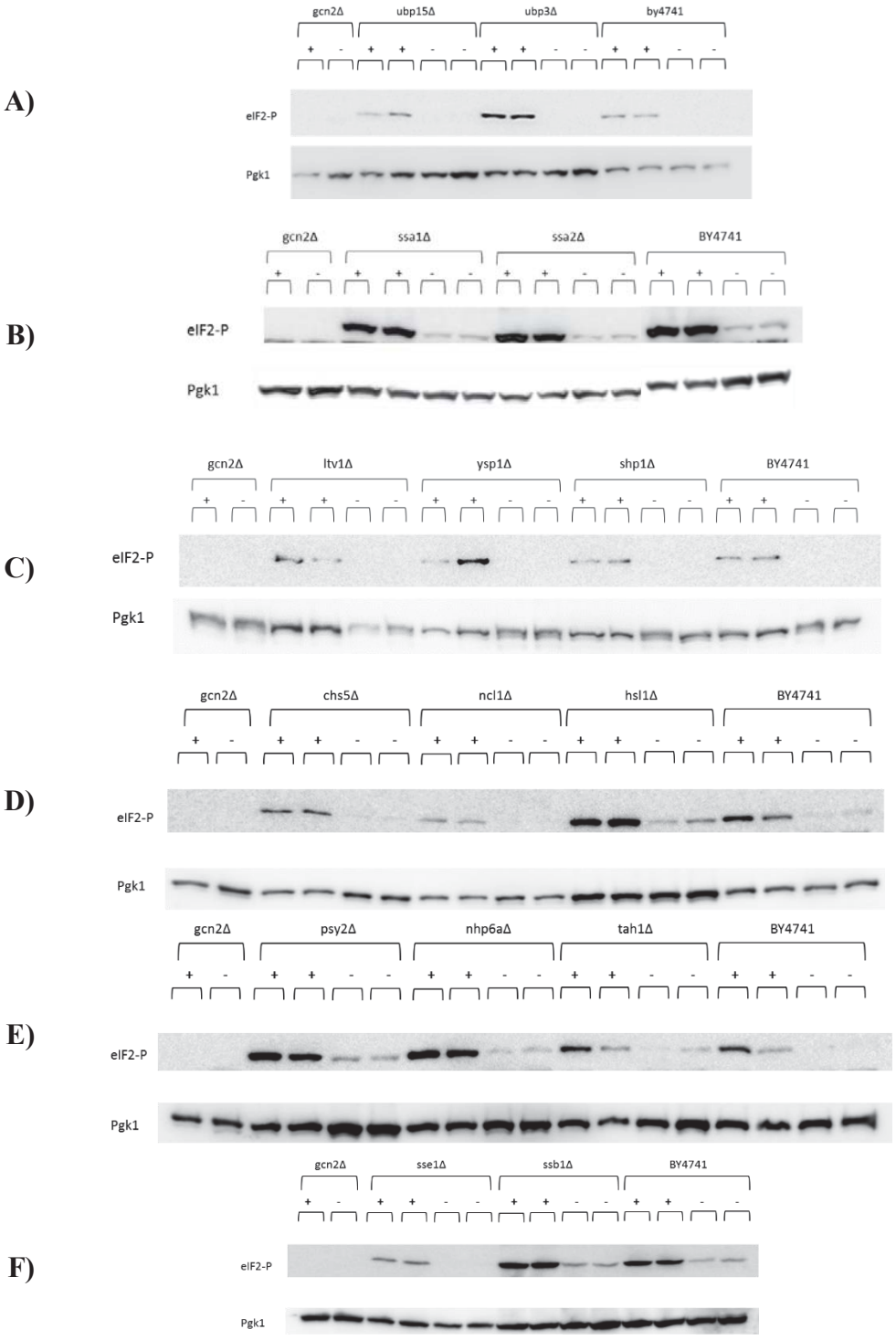


Figure 3.10 eIF2α-P levels in mutants that display sensitivity to SM. Immunoblot of eIF2α-P and Pgk1 from starved (+) and replete (-) indicated strains. Strains were grown in liquid media and starvation was induced by the addition of SM to a final concentration of 1µg/ml for 1 hour where appropriate. All cells were subjected to formaldehyde crosslinking, and lysed. The cell extract was resolved in SDS-PAGE and western blot analysis using eIF2α-P and Pgk1 antibodies.

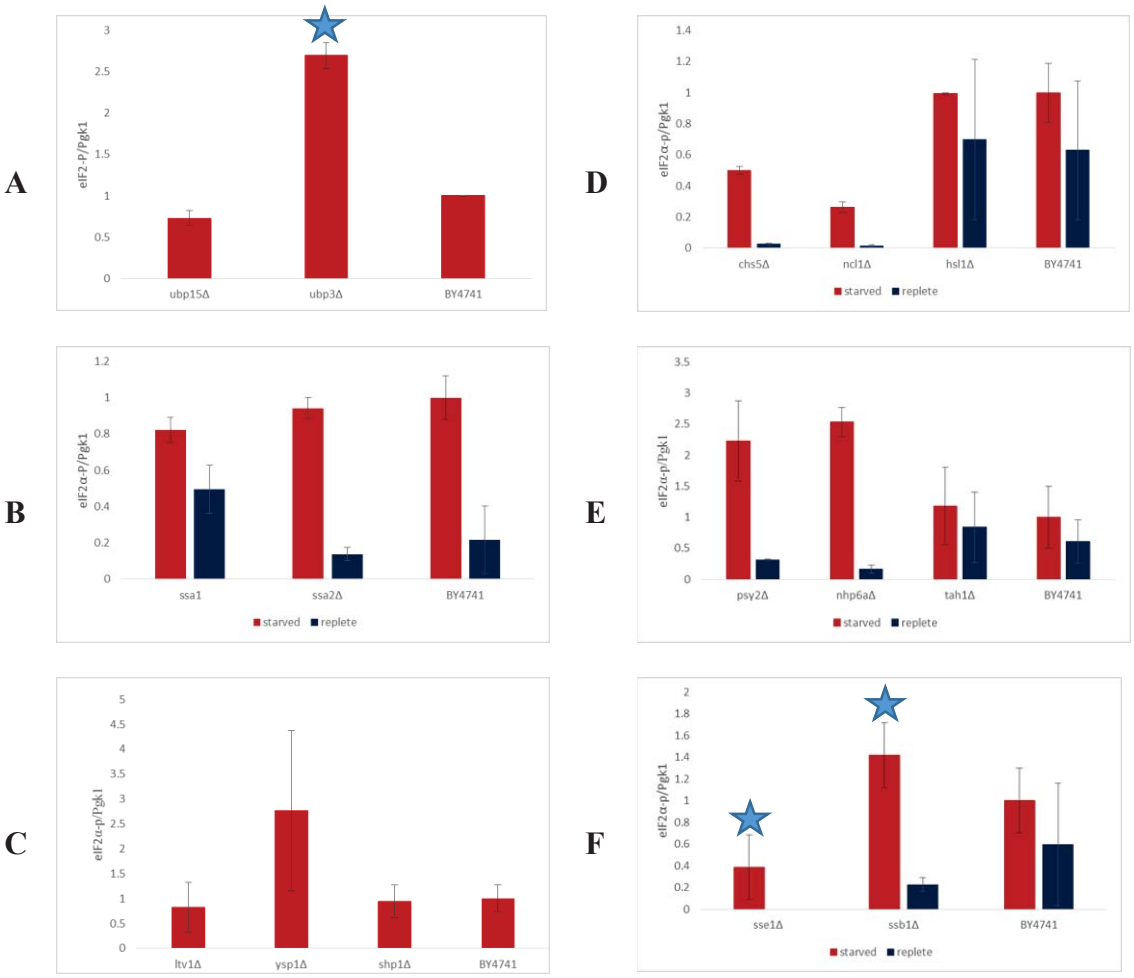


Figure 3.11 comparison of eIF2α-p levels in cultures grown under starved and replete conditions. The eIF2α-p and Pgk1 band signal intensity from the western blot in figure 3.10 was measured using Image J software. The ratio of eIF2α-P to Pgk1 was determined and graphed relative to that of the wild type ratio. Standard error bars were generated using results from two independent cultures. P-values were determined by performing a two-tailed t-test and statistically significant p-values ($P<0.05$) are indicated by a star symbol.

As expected, in the wild type strain (BY4741) the detected levels of eIF2α-P was increased under starvation conditions when compared with the replete culture, signifying activation of Gcn2. Also as expected eIF2α-P was not detected in either the starved or replete cultures of the Gcn2 deletion strain (*gcn2Δ*). The deletion strains that had decreased eIF2α-P levels under starvation conditions compared to the wild type were *ncl1Δ*, *chs5Δ* and *sse1Δ* (Figure 3.11). Additionally the three strains all showed a decrease in eIF2α-P levels under replete conditions. However only one of the deletion strains, *sse1Δ*, had a statistically significant decreased eIF2α-P levels ($P<0.05$) when

starved, according to the results of a two-tailed t-test, in comparison to the wild type (Figure 3.11). Five deletion strains showed an increase in the levels of eIF2α-P when starved compared to the wild type strain, *ssb1Δ*, *ysp1Δ*, *psy2Δ*, *nhp6aΔ* and *ubp3Δ*. Quantification determined that two of these strains *ssb1Δ* and *ubp3Δ* had an increase in starved eIF2α-P levels of statistical significance (Figure 3.11).

In summary, *sse1Δ* was observed to have significantly decreased eIF2α-P levels under conditions of amino acid limitation (Table 3.3) compared with the wild type. This eIF2α-P reduction indicates that Gcn2 is impaired, and that Sse1 may be required for the activation of Gcn2. The *ncl1Δ* and *chs5Δ* strains did have a noticeable decrease in eIF2α-P levels which did not meet the threshold of statistical significance, these proteins however may still be exerting an effect on Gcn2 activation.

Table 3.3 Summary eIF2-P levels SM^s mutant strains

Strain name	ORF systematic name	SM sensitivity	eIF2-P reduction	Characteristics
ubp15Δ	YMR304W	SM^s	No	
chs5Δ	YLR330W	SM^s	Yes	
ncl1Δ	YBL024W	SM^s	Yes	
bre5Δ	YNR051C	SM^s	-	
ssa1Δ	YAL005C	SM^s	No	
ssa2Δ	YLL024C	SM^s	No	
sse1Δ	YPL106C	SM^s	Yes	
ssb1Δ	YDL229W	SM^s	No	↑ eIF2-P
ysp1Δ	YHR155W	SM^s	No	↑ eIF2-P
ltv1Δ	YKL143W	SM^s	No	
hsl1Δ	YKL101W	SM^s	No	
shp1Δ	YBL058W	SM^s	No	
psy2Δ	YNL201C	SM^s	No	↑ eIF2-P
asc1Δ	YMR116C	SM^s	-	
nhp6aΔ	YPR052C	SM^s	No	↑ eIF2-P
ssn8Δ	YNL025C	SM^s	-	
hmo1Δ	YDR174W	SM^s	-	
tah1Δ	YCR060W	SM^s	No	
rad24Δ	YER173W	SM^s	-	
ubp3Δ	YER151C	SM^s	No	↑ eIF2-P

3.2.3 Validation that SM^s is the result of the single gene deletion

There were three deletion strains, *sse1Δ, ncl1Δ* and *chs5Δ* that were found to have reduced eIF2α-P levels under amino acid starvation. Although the decrease in eIF2α-P levels in the *ncl1Δ* and *chs5Δ* strains did not meet the threshold of statistical significance, we chose to still test these proteins, as the eIF2α-P levels were noticeably decreased in these strains under amino acid starvation. Therefore these proteins potentially still have some effect on Gcn2 activity. The reduced cellular eIF2α-P level during starvation may be the consequence of the specific protein being absent in these deletion strains, however there was also a possibility that the observed results were due to an ectopic mutation. If the SM sensitive phenotype and eIF2α-P reduction of the mutant strain was due to the gene deletion, then re-introduction of the gene into the strain should restore the SM^s and eIF2α-P levels to that of the wild type phenotype when subjected to starvation.

Complementation tests were to be performed using the yeast tiling collection available to us within the Sattlegger laboratory. This library comprises overlapping fragments of the entire *Saccharomyces cerevisiae* genome cloned into a high-copy vector (Jones et al., 2008). Ideally complementation should be performed using a vector that only contains the gene of interest, instead of a plasmid that has a genome fragment insert that contains multiple genes in addition to the gene of interest. However the tiling collection is an immediately available resource, not requiring plasmid construction and therefore adequate for initial complementation tests. If the vector with the genome fragment containing the gene of interest cannot rescue the SM^s phenotype of the deletion strain, this would indicate that the SM^s observed was not due to the absent gene, but instead due to an unrelated ectopic mutation.

Three cultures of each deletion strain were transformed with vectors that contained either: no insert (vector alone), an insert containing the entire intact gene of interest ORF or an insert that contains the gene of interest in a truncated form missing sequence from either the 3’ or 5’ terminus (Figure 3.12). The same tiling plasmids were also transformed into a Gcn2 deletion strain as a negative control and the wild type strain was transformed with the plasmid alone. Semi quantitative growth assays were then to be performed as outlined in section 3.2.1. The strains were transformed successfully

unfortunately the growth assays were not able to be performed within the time frame of this thesis.

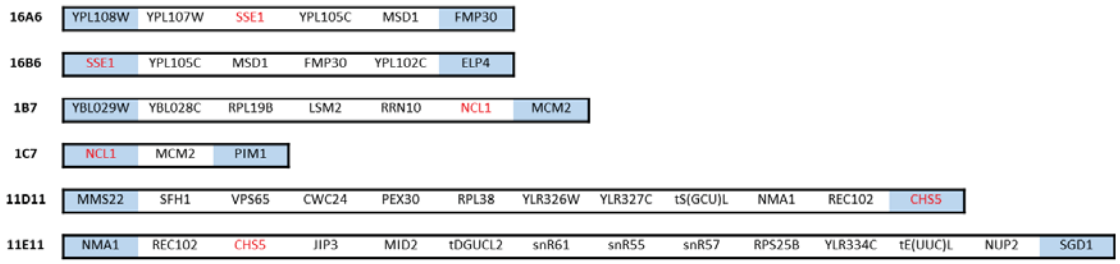


Figure 3.12 Tiling collection plasmid inserts containing genes of interest
The genes contained in the plasmid inserts used in this study from the tiling collection. Genes of interest are shown in red text and truncated genes are shaded blue.

3.3 Screening putative Gcn2 binding proteins to identify inhibitors of Gcn2 activity or activation

The purpose of the research in chapter 3.2 was to identify proteins that positively regulate Gcn2; the aim of this section was to identify potential Gcn2 binding proteins that function in Gcn2 inhibition. To do this we overexpressed the putative binding proteins within cells and screened for SM sensitivity. The reason being that if a protein is a Gcn2 inhibitor then overexpression of that protein would result in stronger Gcn2 inhibition, decreased Gcn2 activation or activity under starvation conditions and as SM sensitive phenotype.

3.3.1 Screening single gene overexpression strains for sensitivity to Sulfometuron Methyl

In order to overexpress the specific proteins of interest, a wild type yeast strain was transformed with plasmid DNA that contained the single gene under a galactose inducible promotor. The plasmids were obtained from an overexpression library available in the Sattlegger laboratory (Hu et al., 2007). Conducting the overexpression screens on all putative gcn2 binding proteins identified in the primary and secondary interactomes (chapter 3.1) would be beyond the time constraints of this research project. The screen was therefore conducted on proteins from the primary interactome that co-precipitated with Gcn2 in two separate studies: Aar2, Hrr25, Prp31, Prp4, Prp6 and Snu114. Cdc48 and Snu66 were unavailable in the overexpression collection. The overexpression collection consists of *E.coli* strains that harbour the plasmids, arrayed in microtiter plates. This meant that to extract one *E.coli* clone, the whole microtiter plate needed to be thawed. Therefore, when thawing plates to extract *E.coli*, clones with tiling plasmids containing the above genes, other clones were extracted that harboured genes coding possible Gcn2-binding proteins if they happened to be on the thawed plates. As a consequence, an additional five proteins were included in the overexpression screen. Reported Gcn2 binding protein Npr1 (Breitkreutz et al., 2010) was also included in this screen.

Plasmids that carried the genes of interest were purified from E-coli. Cultures of Saccharomyces Cerevisiae (strain BY4741) were transformed with the plasmids each containing a single gene expressing a protein of interest from galactose inducible promotor. The wild type (BY4741) was transformed with vector alone as a control for

growth. BY4741 was transformed with Yih1 as the positive control for protein overexpression SM sensitivity, Yih1 inhibits Gcn2 activity when overexpressed by competitively binding Gcn1 (Sattlegger et al., 2004). Growth of these strains in galactose containing media will induce overexpression of the protein. Semi-quantitative growth assays were then performed as described in chapter 3.2.1 on galactose containing media. If Gcn2 activity is impaired by the overexpressed proteins, the strain should display a SM^s phenotype.

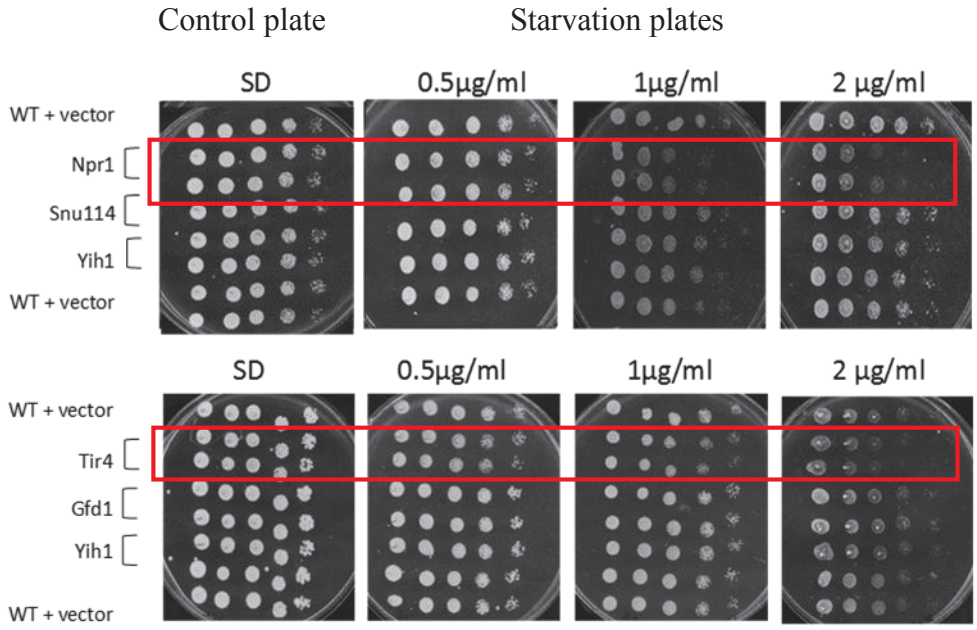


Figure 3.13 Screening to identify proteins that when overexpressed render cells SM^s. Cultures of the control strains and transformed strains were grown to saturation in liquid media. 10 fold serial dilutions of the cultures was performed and 5µl of each dilution was spotted onto SD and SD plates that contained SM at the specified concentration (0.5µg/ml, 1 µg/ml or 2 µg/ml). The plates were then incubated at 30°C and scanned daily for 7- 10 days using a document scanner to record cell growth. For the results of the entire screen see appendix 3.

As expected the wild type strain BY4741 harbouring the vector alone thrived on all of the media used for the semi quantitative growth assays performed including the medium containing SM. The control strain overexpressing Yih1 grew on all media, but displayed a weak SM sensitive phenotype evident in the early stages of strain growth, as expected. Interestingly, over expression of Npr1 and Tir4 lead to a pronounced sensitivity to SM (Figure 3.13).

3.3.2 Screening overexpression strains with SM^s for impaired Gcn2 function

To determine whether the SM^s triggered by the overexpression of Npr1 and Tir4 was due to reduced Gcn2 function, the levels of eIF2-P were investigated as described in chapter 3.2.2. The only difference were that the strains were grown in media that contained galactose to induce protein overexpression and uracil was omitted from the media to maintain the plasmid, which harboured a uracil selectable marker.

As expected, in the wild type strain (BY4741) the detected levels of eIF2α-P was increased under starvation conditions when compared with the replete culture (Figure 3.14, lanes 5, 6, 7, 8, 13, 14, 15 and 16) signifying that Gcn2 had been activated. Also, as expected, eIF2α-P was not detected in the sample from the Gcn2 deletion strain (gcn2Δ lane 17).

The eIF2α-P levels in the starved sample from the Tir4 overexpression strain were comparable to the wild type strain when quantified (lanes 9, 10, 13 and 14), however the replete sample had significantly increased eIF2α-P levels (~15 fold) compared to the wild type (lanes 1, 2, 5 and 6). The Npr1 overexpression strain showed a 2.4 fold increase in levels of eIF2α-P under starvation conditions when compared to the wild type (lanes 11, 12, 13 and 14), and the levels of eIF2α-P in the replete sample from the Npr1 overexpression strain were almost identical to that of the wild type strain (lanes 3, 4, 5 and 6).

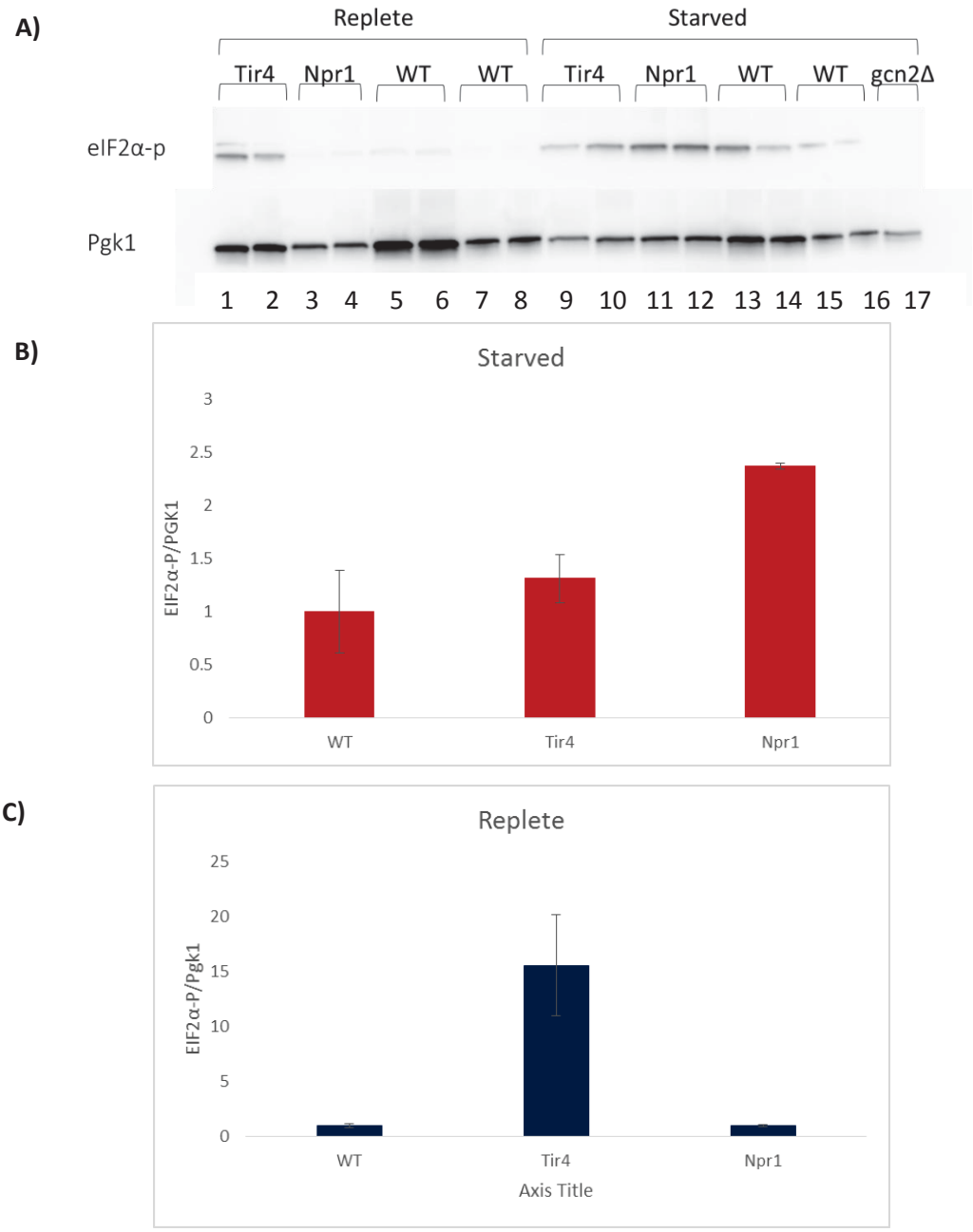


Figure 3.14 comparison of eIF2α-p levels in starved and replete cultures of SM^s gene overexpression strains with the wild type. A) Immunoblot of eIF2α-P and Pgk1 from starved (+) and replete (-) indicated strains. Strains were grown in liquid media and starvation was induced by the addition of SM to a final concentration of 1µg/ml for 1 hour where appropriate. All cells were subjected to formaldehyde crosslinking, and lysed. The cell extract was resolved in SDS-PAGE and western blot analysis using eIF2α-P and Pgk1 antibodies**. B)** The eIF2α-p and Pgk1 band signal intensity from the starved sample bands in the western blot in figure 3.14A were measured using Image J software. The ratio of Pgk1 to eIF2α-P was determined and graphed relative to that of the wild type ratio. Standard error bars were generated using results from two independent cultures **C)** The eIF2α-p and Pgk1 band signal intensity from the replete sample bands in the western blot in figure 3.14A were measured using Image J software The ratio was determined standard error bars were generated as for figure 3.14B.

3.4 Establishment of a his_6 tag mediated Gcn2 affinity purification procedure.

The binding of deacylated tRNA to the HisRS domain of Gcn2 is the fundamental signal for Gcn2 activation (Wek, Jackson, & Hinnebusch, 1989a; S. A. Wek, S. Zhu, & R. C. Wek, 1995a), however Gcn2 activity is also governed by binding of Gcn2 to other proteins such as Gcn1 and eEF1A. (Doerks, Copley, Schultz, Ponting, & Bork, 2002a; Visweswaraiah et al., 2011) The recent discovery of eEF1A and Npr1 as Gcn2 binding proteins indicates that there are likely to be additional undiscovered proteins that also bind to Gcn2 and regulate its activity. Characterisation of Gcn2 interactions with proteins and tRNA, and the comprehensive identification of proteins binding to Gcn2, requires the affinity purification of Gcn2 along with its binding partners. This is a procedure which has thus far been difficult to perform effectively and reproducibly (Sattlegger laboratory, unpublished). The aim here was to further optimize a Gcn2 purification procedure

Histidine has a high affinity for metal ions including Ni^{2+} and Co^{2+}. These metal ions when immobilised on a matrix readily capture proteins that are expressed with a tag composed of repeated histidine residues, such as the his_6 affinity purification tag, consisting of 6 consecutive His residues. The his_6 epitope tag is small in size and is not strongly charged, it is therefore unlikely to negatively affect the function of the tagged protein or its interaction partners. Histidine binds to specific metal ions via its imidazole side chain. Because imidazole is structurally similar to the his_6 tag and binds Ni^{2+} with a similar affinity, captured proteins can be released from the matrix using buffers containing a competitive concentration of imidazole (Crowe et al., 1994; Porath, 1992).

In this study a plasmid was used that contains N-terminally Flag-his_6 tagged Gcn2 under a galactose inducible promotor. This promotor enabled Gcn2 overexpression to be induced using Galactose. Increased Gcn2 levels in the cell were used with the intention of driving weak and transient interactions, to increase the probability of detecting the corresponding Gcn2-binding proteins. The Gcn2 construct that we used also contained a Flag tag, however we did not use this tag for purification as we have found previously that it is not suitable for Gcn2 purification conditions aimed to preserve protein-protein interactions (Sattlegger lab unpublished). Therefore, in this thesis the aim was to test the suitability of the his_6 tag for Gcn2 purification. For this, it would have been optimal to use a Gcn2 construct that contains only the his_6 tag, however this was not available.

The advantage of the available construct was that it had already been characterised to be functional (J. Dong et al., 2000). This plasmid was introduced in a Gcn2 deletion strain, meaning that the resulting transformant only harboured the tagged version of Gcn2 in the cells. In addition, a Gcn2 allele was used that contained the E803V substitution in Gcn2. The E803V mutation is known to increase Gcn2's affinity to tRNA by weakening the auto-inhibitory PK domain interaction. Due to the enhanced tRNA binding this Gcn2 allele is constitutively active (Qiu et al., 2001b). The E803V strain was used for some of the optimisation as it is intended for future use in tRNA binding assays.

A preliminary protocol for Gcn2 affinity purification was provided by a lab member, which required further optimisations for efficient Gcn2 binding and elution.

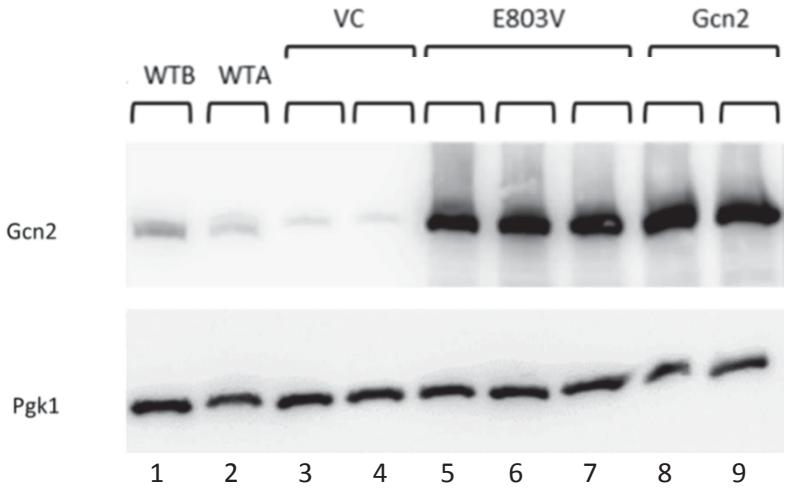
3.4.1 Verification of Gcn2 expression

As overexpression of Gcn2 only occurs in the strains upon induction by galactose, the strains were checked to verify that Gcn2 was overexpressed when cells were grown in media containing galactose. For this, the strains were cultured in minimal (SD) liquid medium containing galactose as sole carbon source. The cells were harvested in early exponential phase, at an OD of 1. Cell extracts were generated and the extracts were subjected to SDS-PAGE and western blot analysis was performed using antibodies against Gcn2. The housekeeping protein Pgk1 was also detected as the loading control.

The Gcn2 signal was significantly more intense in the galactose induced his_6-Gcn2 containing strain (Figure 3.15A, lanes 8 and 9) when compared with the isogenic wild type extracts in which Gcn2 is expressed from its endogenous promotor (Figure 3.15A, lanes 1 and 2). There was a slight band evident in the vector control lanes at the expected Gcn2 molecular weight location, this was identified previously during in house studies as an antibody background band and was accounted for during quantification (Figure 3.15A, lanes 3 and 4). The signal intensity of the bands representing the quantity of Gcn2 and Pgk1 were measured using Image J software. Quantification of the signals indicative of Gcn2 expression was performed, and the signal intensity was divided by that of the Pgk1 signal. The results showed that Gcn2 expression was approximately 20 fold higher in the overexpression strain than in the

wild type. His_6-Gcn2-E803V expression was about 10 fold higher than the native Gcn2 level. Taken together, these results indicated that the strains were effectively overexpressing Gcn2 (Figure 3.15 B).

A)



B)

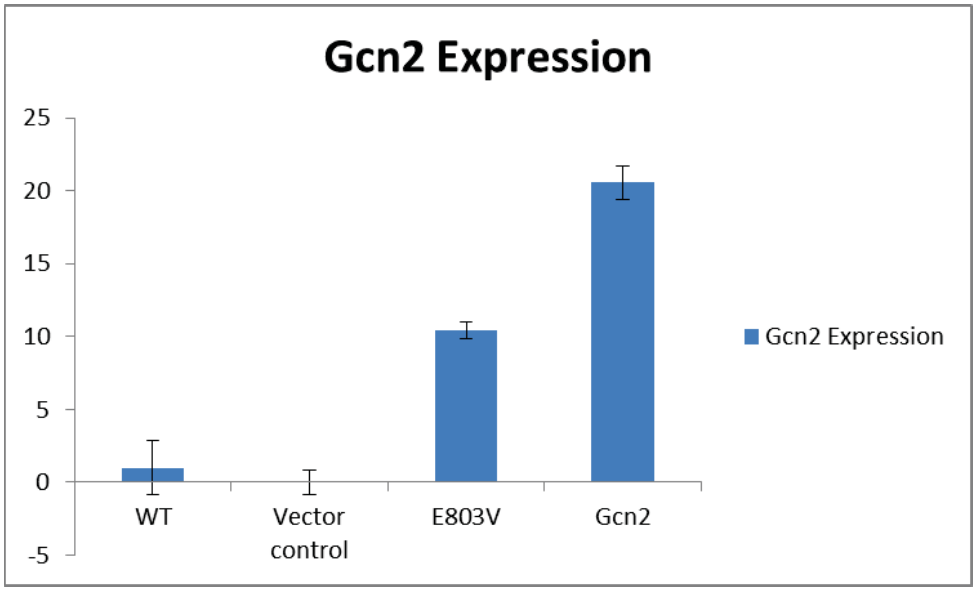


Figure 3.15 Determining the Gcn2 expression levels in yeast cells. Immunoblot of Gcn2 and Pgk1 expression in wild type (WT) and *gcn2Δ* stains which contain plasmids with either his_6-Gcn2, his_6-Gcn2-E803V or the vector alone (VC). The cultures were grown in galactose containing minimal medium. Cell extract was prepared using NaOH-mediated lysis and resolved in SDS-PAGE. Proteins were transferred to PVDF membrane and detected using Gcn2 and Pgk1 antibodies. B) The Gcn2 and Pgk1 band signal intensity from the western blot analysis using corresponding antibodies was measured using Image J software. The ratio of Gcn2 to Pgk1 was determined and graphed in comparison relative to the wild type ratio. Standard error bars shown were generated from the results of two independent colony cultures of each strain

In the above analysis NaOH was used to lyse cells. This is a fast lysis method reducing the probability of protein degradation due to the liberation of proteases from the organelles during lysis. Together with the fact that this procedure did not involve high rpm centrifugation steps that may sediment large protein complexes, it allowed us to gauge how much Gcn2 was present in the cells. The protocol to purify Gcn2 from cell extract cannot utilise alkaline lysis but instead requires mechanical lysis, therefore the next step was to check for Gcn2 presence in cell extracts that had been prepared using mechanical cell lysis. The cells were grown in galactose SD and harvested, before being lysed mechanically using glass beads. Again, the cell extract was resolved on SDS-PAGE, the proteins were then transferred to PVDF membrane and Gcn2 and Pgk1 were detected. Full length Gcn2 was detectable as a clear band at the expected molecular weight of 180kDa in the Gcn2-overexpression strain (Figure 3.16, lanes 1 and 2) but not sample containing endogenous Gcn2 levels (lanes 5 and 6). The latter is likely due to the Gcn2 levels being below the detection limit. Even though the endogenous Gcn2 could not be detected, the fact that in the overexpression samples Gcn2 could be readily detected, strongly suggests that the cell extract generated by mechanical lysis contained non-degraded Gcn2 and was suitable for a subsequent Gcn2 purification procedure.

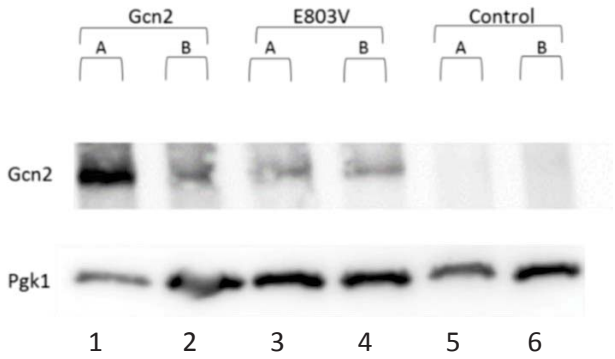


Figure 3.16 Gcn2 is detectable in extracts from Gcn2-overexpression strains generated by mechanical lysis. Immunoblot of showing Gcn2 and Pgk1 detectable in his_6-Gcn2, his_6-Gcn2-E803V and control strains. 'A' and 'B' indicate independent colony cultures of each strain. The cultures were grown in SD with galactose. Cell extract was prepared using glass bead mediated mechanical lysis and resolved on SDS-PAGE. Proteins were transferred to PVDF membrane and detected using an antibody against Gcn2 and Pgk1.

3.4.2 Optimisation of Gcn2 purification using iMAC resin

A trial purification (pulldown) was performed. 300ml cultures of the strains harbouring his_6-Gcn2 or the vector alone were grown to exponential phase and cell extract was prepared by mechanical lysis. The total protein concentration in each cell lysate was estimated using the Bradford method. Samples were prepared from each strain that contained 1100µg of total protein in a volume of 440µl. The samples were then pre-cleared by incubating them for 1 hour with agarose beads (Sepharose, GE Healthcare). This procedure removes any proteins from the lysate that may a high non-specific affinity to agarose. 400µl of the pre-cleared sample was then transferred to 50µl (bead volume) pre-washed iMAC resin and incubated rotating at 4°C for two hours. A sample of the supernatant was then retained for analysis. The iMAC resin (Biorad) is a matrix bearing immobilised Ni^{2+} capable of binding the his_6 tag. To remove proteins that may have bound non-specifically, the iMAC beads were washed three times with buffer containing 30mM imidazole.

The Gcn2 was then released from the resin, by suspending the beads in Laemmli loading dye and boiling them for ten minutes at 95°C. The samples were subjected to SDS-PAGE, western blotting and Gcn2 detection. The immunoblot revealed that Gcn2 had successfully bound to the iMAC resin (Figure 3.17, lanes 9 and10). No bands were detected in any of the vector control samples, as expected (lanes 3, 4, 7, 8, 11 and 12). There was an inadequate quantity of input control and supernatant samples resolved in the gel for Gcn2 to be visualised in the samples at the expected molecular weight, however bands were detected at a lower molecular weight in these samples which may be fragments of degraded Gcn2 or unspecific antibody binding (lanes 1, 2, 5 and 6).

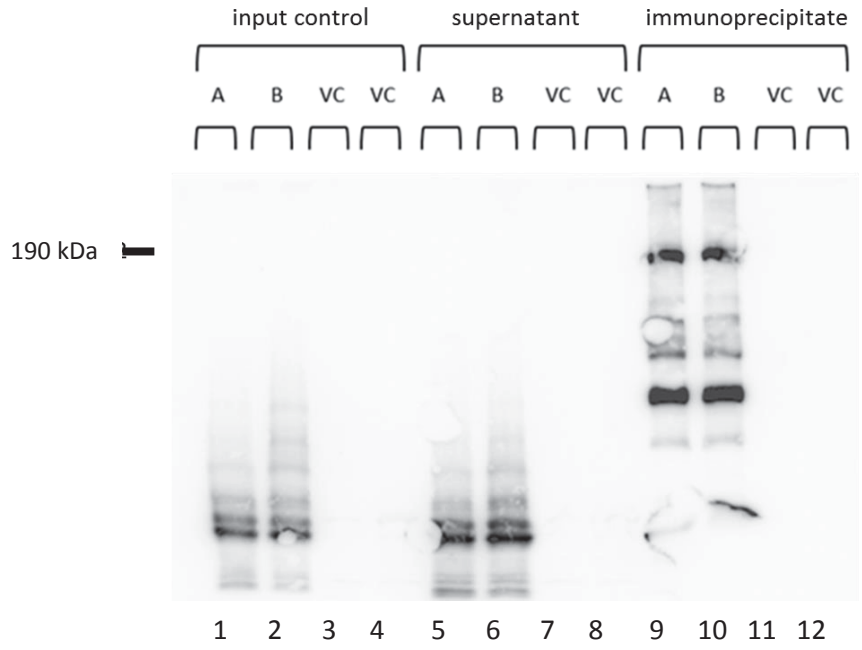


Figure 3.17 Purification of Gcn2 from cell extract. Cultures of the indicated strains were grown to exponential phase and cell extract was prepared using glass bead lysis. A and B are independent colony cultures of the his_6-Gcn2 expressing strain, (VC) two independent colonies of the vector control strain were also used. 1000µg samples were subjected to iMAC mediated protein purification. Gcn2 was then released from the resin, by suspending the beads in SDS-PAGE loading dye and heating them for ten minutes at 95°C. The samples were subjected to SDS-PAGE, western blotting and gcn2 was detected using anti Gcn2 antibody. The band at 190 kDa indicates the expected position of Gcn2.

Next we aimed to follow the preliminary protocol to be used for detecting Gcn2-binding proteins. Here, in order to preserve and stabilise protein interactions for Gcn2 binding partner co-precipitation, we wanted to perform the purification process after treating the cells with formaldehyde immediately prior to harvesting. Formaldehyde instantly crosslinks protein-protein interactions. An optimum sample for Mass spectrometric analysis also requires Gcn2 to be eluted from the iMAC beads rather than treating the beads with loading dye. Thereby, releasing only protein complexes linked to the resin via a His tag, as opposed to all of the proteins present on the beads including the ones that are bound non-specifically. Previous studies in the Sattlegger lab (unpublished) had shown that 1% formaldehyde was optimal for complex stabilisation. Therefore cells were grown and harvested as described before, except that prior to harvesting, the cells

were treated with 1% HCHO at 4°C for one 1h. In addition, bound Gcn2 complexes were eluted using 250mM imidazole for three consecutive elution steps. .

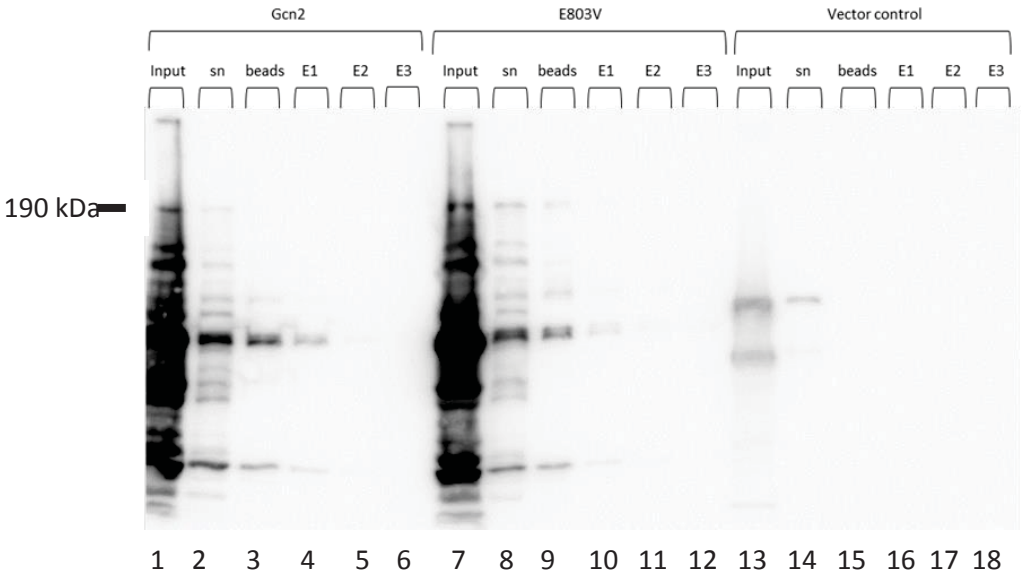


Figure 3.18 Attempted Gcn2 purification using formaldehyde crosslinked samples and imidazole elution. Cultures of the indicated strains were grown to exponential phase and cell extract was prepared using glass bead-mediated lysis. 1000µg of total protein was subjected to iMAC-mediated protein purification. Gcn2 was eluted from the beads using 250mM Imidazole. The samples were subjected to SDS-PAGE, western blotting and Gcn2 was detected using anti Gcn2 antibody. The band at 190 kDa indicates the expected position of Gcn2.

Aliquots of the input, supernatant, and the elution steps were subjected to SDS-Page and western blotting. In addition the beads were suspended in loading dye and heated to release any bound Gcn2 that may not have been eluted during the elution steps. Full length Gcn2 was present in the input control of the his_6-Gcn2 and the his_6-Gcn2-E803V expression strains (Figure 3.18, lanes 1 and 7). Faint bands indicate that there was also some Gcn2 in the supernatant of these strains (lanes 2 and 8), Gcn2 presence in the supernatant indicates that not all of the sample Gcn2 bound to the iMAC resin during incubation. There was no indication of Gcn2 in the vector control strain samples (lanes 13 to 18), however some unspecific signals were noted in the input control and supernatant lanes. The absence of Gcn2 in the elution samples and on the beads indicated that Gcn2 may have either not bound to the beads or it may have been lost during the washing steps (lanes 3, 4, 5 and 6). The washes were performed using buffer

with 30mM imidazole to release any proteins bound non-specifically to the resin. To determine whether Gcn2 had been lost during the washing stage of the protocol the first two wash steps were resolved on SDS-PAGE, and western blot analysis was performed. Full length Gcn2 was not detected in the washing buffer. Bands were detected at molecular weights lower than that of Gcn2 which are likely to be Gcn2 fragments as they were absent in the vector control strain (Figure 3.19).

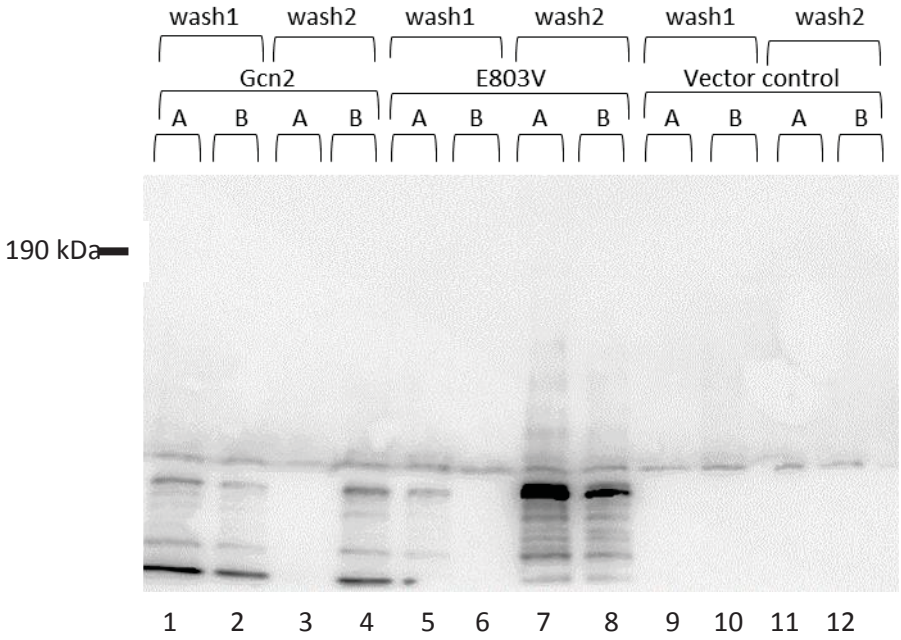


Figure 3.19 Immunoblot wash buffer from unsuccessful pull-down assay. iMAC resin was subjected to three wash cycles using 500µl buffer containing 30mM Imidazole. Two of the wash buffer samples from each indicated strain was resolved by SDS-PAGE and Immunoblotted using antibodies against Gcn2. A and B indicate independent colony cultures of each strain**.** The band at 190 kDa indicates the expected position of Gcn2.

In order to prevent Gcn2 degradation during the iMAC purification, multiple repeat attempts at the procedure were performed. During these attempts basic stepwise troubleshooting was performed, which included the use of newly prepared reagents and strains, increasing the concentration of protease inhibitors and adjusting the buffer composition. During all of these attempts Gcn2 degradation remained a problem, due to visualising only degradation products and no Gcn2 in the western blot analysis, our results were difficult to interpret (Figure 3.20).

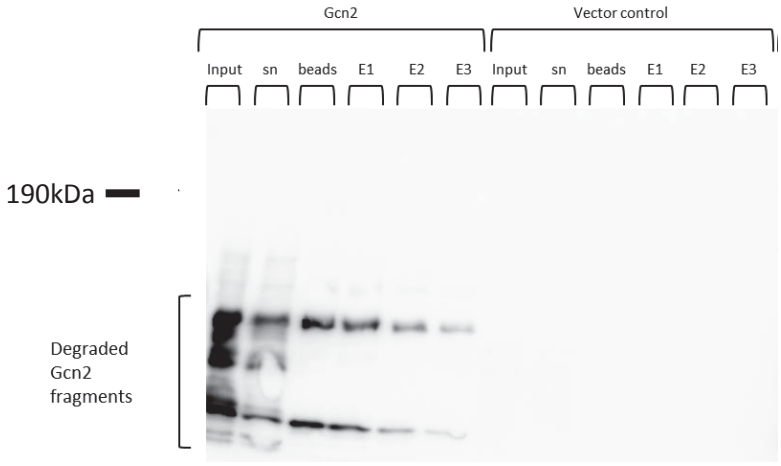


Figure 3.20 Representation of typical results from iMAC pull-down assays for Gcn2 purification. Cultures were grown, cell extract was prepared, and samples were subjected to iMAC protein purification, elution, SDS-PAGE and immunoblotting as described in figure 3.18.

Troubleshooting was complicated by the fact that degradation appeared to be sporadic. Many experiments resulted in only degradation products while intermittent experiments were performed where Gcn2 was not degraded. It was posited that laboratory chemical stock powders may be potentially contaminated with proteases such as Proteinase K. This enzyme is used in the laboratory in powder form, and is inherently stable. Gcn2 has a short half-life and degrades easily (Wei et al., 2015), even trace amounts of a broad spectrum proteinase could be sufficient to lead to complete Gcn2 degradation. Once these chemical stocks were exchanged it was possible to perform some additional optimisations, as discussed below.

3.4.3 Formaldehyde crosslinking optimisation

In house experiments investigating low abundant protein complexes utilised crosslinking using a final concentration of 1% formaldehyde at 4°C. Crosslinking using a high formaldehyde concentration can lead to the aggregation of large non-physiological aggregates that may be lost during the procedure of generating cell extracts (Wong, 1991). The aim was to determine the optimal concentration required to retain Gcn2 in the cell extract while stabilising the interactions formed between Gcn2 and its binding partners.

Exponentially growing cultures were treated with formaldehyde at concentrations of 1%, 0.5%, 0.3%, 0.1% and 0% for 1 hour at 4°C and cell extracts were prepared. The protein concentration was determined using the Bradford method and duplicate samples containing 100µg of total protein were mixed with SDS-PAGE loading dye. One set was denatured for ten minutes at 95°C and the other set at 65°C. Denaturing the sample at 95°C results in reversal of the majority of the formaldehyde derived bonds, while the crosslinks should be predominantly preserved when the sample is boiled at 65°C. At 95°C Gcn2 should therefore be detected at the expected molecular weight and represent the total quantity of Gcn2 in each extract. At 65°C Gcn2 should be still bound to other proteins and therefore should be observed at a higher molecular weight because it migrates slower through the gel. Gcn2 should also be detected at the expected molecular weight demonstrating the efficacy of the formaldehyde crosslinking at each concentration.

Following denaturing at 95°C, there were strong signals for Gcn2 at the expected molecular weight of 180kDa in all of the samples. (Figure 3.21A, lanes 6 to 10). There was also a faint band above the 250kDa marker which is most likely a background band because the same band can be observed in the control lane. In the set that was denatured at 65°C there are bands at varying intensities at the expected molecular weight of Gcn2 in all of the samples, bands were also detected above the molecular marker of 250kDa representing Gcn2 in complexes too large to migrate through the acrylamide gel (lanes 1 to 5). The band signal intensities of the band at 180kDa and 250kDa were quantified utilising Pgk1 as a loading quantity control and standardised to the non-crosslinked sample (0%).

Quantification of the signal intensities indicated, as expected, that as the formaldehyde crosslinking concentration increased the quantity of Gcn2 in large complexes at the high molecular weight increased (Figure 3.21 B). In agreement with this, quantification of the bands representing not-crosslinked Gcn2 indicated that the amount of non-crosslinked Gcn2 in the samples decreases with an increasing formaldehyde crosslinking concentration (Figure 3.21C). At 1% formaldehyde concentration the majority of Gcn2 exists in large complexes. 0.5% or 0.3% formaldehyde concentrations were chosen as the most likely candidates to afford the best outcome, for sufficient crosslinking of Gcn2 with interacting proteins while minimising Gcn2 loss due to extensive protein conjugation.

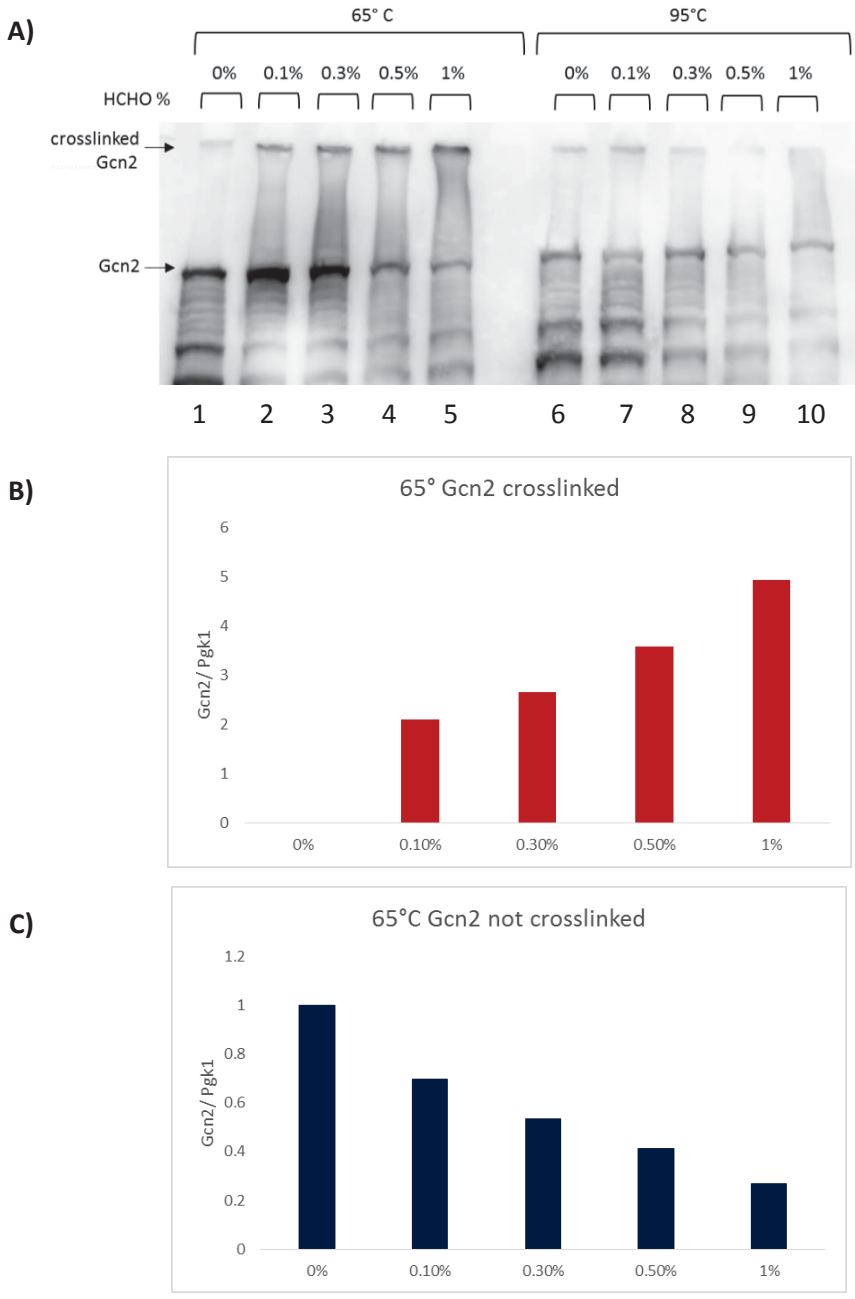


Figure 3.21 Formaldehyde crosslinking optimisation. **A)** Cultures were treated with formaldehyde at concentrations of 1%, 0.5%, 0.3%, 0.1% and 0% and cell extracts were prepared. The protein concentration of each extract was estimated and two sets of each cell extract were mixed with SDS-PAGE loading dye and denatured for ten minutes, one set at 95°C and the other at 65°C. The samples were resolved by SDS-PAGE and immunoblotted with antibodies against Gcn2 and Pgk1. **B)** The Gcn2 and Pgk1 band intensities of the bands above 250kDa in the 65°C denatured sample from the western blot figure 3.21A were measured using Image J software. The ratio of Gcn2 to Pgk1 was standardised to the ratio of the non- crosslinked sample (0%). **C)** The Gcn2 and Pgk1 band intensities of the 180kDa bands in the 65°C denatured sample from the western blot figure 3.21A were measured using Image J software. The ratio of Gcn2 to Pgk1 was standardised to the ratio of the non- crosslinked sample (0%).

It was next investigated whether 0.3% or 0.5% formaldehyde crosslinking is most suitable for the iMAC mediated purification of Gcn2. Gcn2 purification was performed as described in chapter 3.4.2 using extracts that were treated with 0.5% or 0.3%, formaldehyde prior to harvesting. To reduce the rate of Gcn2 degradation, in these optimisation trials the elution steps were omitted and instead the precipitates removed from the resin by treatment with loading dye at 95°C for 10 minutes.

The amount of precipitated Gcn2 decreased with the increased concentration of formaldehyde used for crosslinking. Treatment of the cells with 0.5% formaldehyde resulted in a significantly decreased signal compared to the 0.3% and 0% formaldehyde treated cells (Figure 3.22, lanes 1, 2 and 3). Therefore 0.3% formaldehyde treatment is optimal for the iMAC-mediated Gcn2 purification. The abundant protein Pgk1 was not found in the precipitates (lanes 1 to 6). Given that Pgk1 does not form a complex with Gcn2 and that Pgk1 is a listed contaminant protein in affinity purification studies (Ho et al., 2002) this suggested that the purification procedure had successfully reduced sample contaminant proteins.

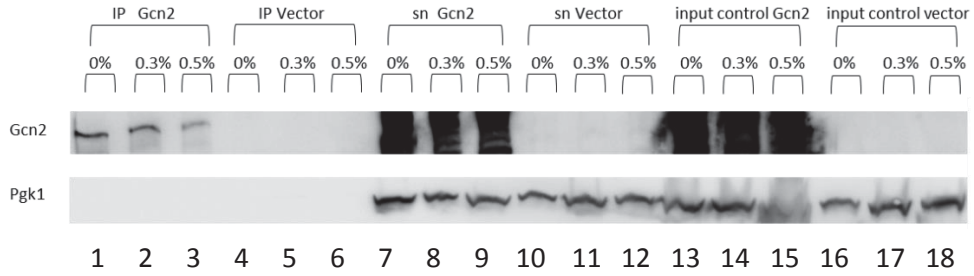


Figure 3.22 Purification of Gcn2 from cell extract. Cultures of his_6-Gcn2 overexpressing strain and the vector alone strain were grown to exponential phase, crosslinked with formaldehyde at the indicated concentration (%), and cell extract was prepared using glass bead-mediated lysis. 1000µg total protein of each sample was subjected to iMAC-mediated protein purification. Gcn2 was then eluted from the beads by incubating them for 10 minutes at 95°C in SDS-PAGE loading dye. The samples were subjected to SDS-PAGE, western blotting and immunoblotted using antibodies against Gcn2 and Pgk1.

3.4.4 Protease inhibitor composition does not reduce Gcn2 degradation

Investigations into Gcn2 derived from human cell lines showed that the Gcn2 has a short half-life (Wei et al., 2015) and is therefore easily degraded. During the purification procedure measures were taken to reduce the amount of Gcn2 degradation that occurred within our samples. Despite this, a substantial amount of degraded Gcn2 was detected in all of the samples. We wanted to determine whether the protease inhibitor composition used in the purification procedure could be improved to reduce Gcn2 degradation. Two forms of protease inhibitors were trialled in the buffer used during purification process, the first was the commercially prepared protease inhibitor tablet (Roche), which is dissolvable in buffer and contains a mixture of protease inhibitors. This is the standard procedure used in the Sattlegger lab. The context of this tablet is proprietary. Previously we had trialled doubling of the concentration of these inhibitors and this did not have an effect on the degradation in the samples. The protease inhibitor mixture in the alternative buffer we trialled was composed of individually purchased powder inhibitors prepared and added to the buffer with a final concentration of 1mM PMSF, 10µg/ml Pepstatin, 1µg/ml Aprotinin and 1µg/ml Leupeptin. Comparatively there was no reduction in the amount of Gcn2 degradation in either of the samples using tablet inhibitors or individual inhibitors (Figure 3.23, lanes 1, 2, 3, 7, 8, and 9).

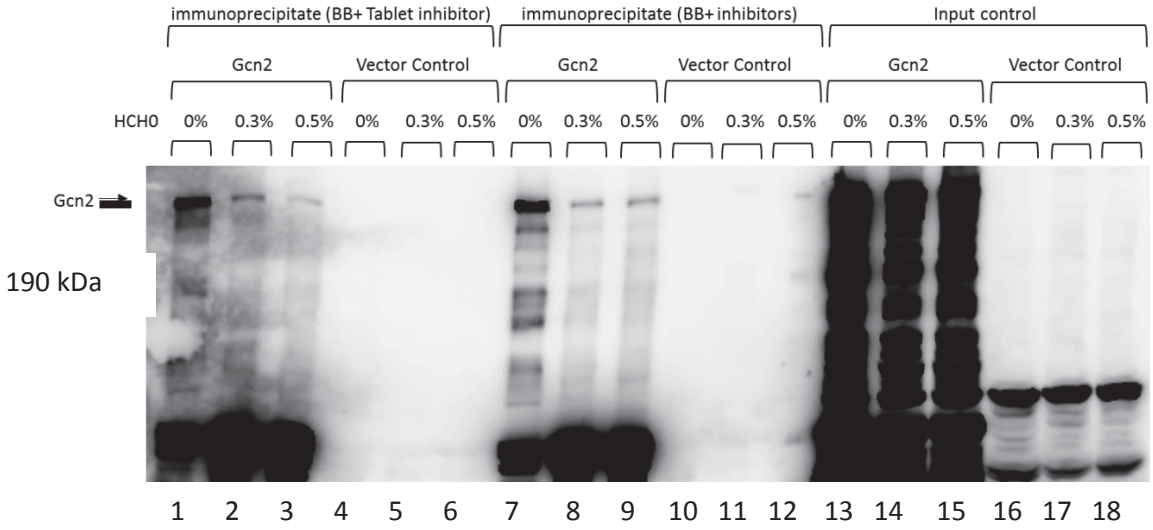


Figure 3.23 Comparison of Gcn2 purifications performed in presence of different protease inhibitor compositions. Cultures of his_6-Gcn2 overexpression strain and the vector alone strain were grown to exponential phase, treated with 0, 0.5 or 0.3% formaldehyde and cell extract was prepared using glass bead mediated lysis with breaking buffer with one of two alternate protease inhibitor preparations. 1000µg total protein containing samples were subjected to iMAC protein purification. Gcn2 was then eluted from the beads by heating for 10 minutes at 95°C in SDS-PAGE loading dye. The samples were subjected to SDS-PAGE, western blotting and immunoblotted using antibodies against Gcn2. The 190 kDa marker indicates the expected position for Gcn2.

3.4.5 Gcn2 iMAC purification with Imidazole elution

The optimal formaldehyde concentration for purifying Gcn2 containing complexes for Gcn2 was determined to be 0.3% and successful purification had been performed by eluting Gcn2 from the iMAC beads using heat treatment in Laemmli loading dye. Purification for the purposes of mass spectrometric analysis however required a sample of Gcn2 to be purified using Imidazole elution. To do this a pulldown was performed as described in section 3.4.2. After incubation of the cell lysate with the iMAC resin, the beads were washed five times with buffer containing 30mM imidazole and elution was performed three times using 200µl buffer containing 250mM imidazole. Gcn2 was detected in all three elution fractions at the expected molecular weight in the his_6-Gcn2 samples (Figure 3.24, lanes 5, 7 and 9). Gcn2 was not detected in any vector control sample (lanes 2, 4, 6, 8 and 10). Pgk1 was detected in the Input control and supernatant of all input control and supernatant samples (lanes 1 to 4), Pgk1 is an abundant protein within the cell that is not known to interact with Gcn2, the absence of Pgk1 in the

eluates infers that the Gcn2 has been purified with no detectable contamination. In order to confirm that Gcn2 had co-purified bound to interacting proteins, we also looked for known Gcn2 binding partner Gcn1, ideally Gcn1 would be detected in the samples co-purified with Gcn2. Gcn1 was detected in the input control and supernatant in all of the samples however was absent in the elution samples (Figure 3.24, lanes 5, 7 and 9). This may have been because Gcn1 is not an abundant protein within the cell and co-purified levels may therefore not be present in the eluates at a detectable level.

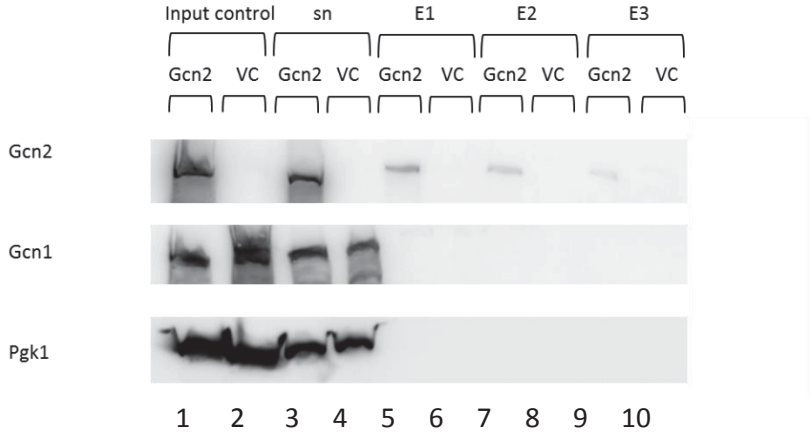


Figure 3.24 iMAC mediated Gcn2 purification with imidazole elution 300ml cultures of his_6-Gcn2 expressing and vector control strains were grown to exponential phase, treated with 0.3% formaldehyde and cell extract prepared using mechanical lysis. 1000µg total protein of each sample was precleared and subjected to iMAC-mediated precipitation of Gcn2. Gcn2 was eluted from the beads using 250mM Imidazole. The samples were subjected to SDS-PAGE and immunoblotted using antibodies against Gcn2, Gcn1 and Pgk1.

3.4.6 Large scale purification of Gcn2 and bound interaction partners

A large scale purification was performed for the purposes of obtaining a purified sample with an amount of Gcn2 that allows the detection of co-purifying Gcn1, and to obtain enough sample for mass spectrometry mediated detection of Gcn2-binding proteins. The strains utilised for this procedure were the his_6-Gcn2 expression strain and the vector control strain. Four sets of each strain were grown to exponential phase in 300ml of SD-galactose. The cell extract was prepared immediately for one of the sets (0%), the other set was treated with 0.3% formaldehyde before cell extract preparation. The cell extracts each with a total protein concentration of 1200µg were pre-cleared by incubating with

Sepharose beads for 1 hour, supernatant containing 1000µg of protein was then incubated with 50µl of iMAC beads for 2 hours. For each sample set four individual pulldowns were performed, the beads were then pooled in one tube before commencing the elution steps. Aliquots of the eluates were then subjected to SDS-PAGE and immunoblotting. Gcn2 was detected at the expected molecular weight, in the input control and the three eluates (Figure 3.25, lanes 1, 2, 5, 6, 9, 10, 13 and 14). As expected, there was no Gcn2 signal detected in the any of the vector control lanes (lanes 3, 4, 7, 8, 11, 12, 15 and 16). Detection of Gcn1 was performed to determine whether this known Gcn2 binding partner had co-precipitated with Gcn2. Gcn1 was identified in the first and second elution steps of the non-crosslinked samples, and in the first elution step of the 0.3% crosslinked samples (lanes 5, 6 and 9). After a long exposure time Gcn1 was evident the Vector control 0% E1 sample (lane 7), most likely due to unspecific binding of Gcn1 to the iMAC resin. However, there is far more Gcn1 present in the samples containing Gcn2 than in the vector alone control, strongly suggesting that Gcn1 co-precipitated with Gcn2. Pgk1 was detected only in the input control (lanes 1, 2, 3 and 4) and was not detected in any of the eluates indicating that general unspecific binding of proteins to the iMAC beads was minimal (Figure 3.25). Together this suggested that Gcn2 was purified successfully along with its binding partners. The remaining samples were resolved on SDS-PAGE and the gel was then sent to the University of Otago for mass spectrometric analysis. Unfortunately this analysis was not successful, we were not able to repeat the purification procedure within the time frame of this thesis.

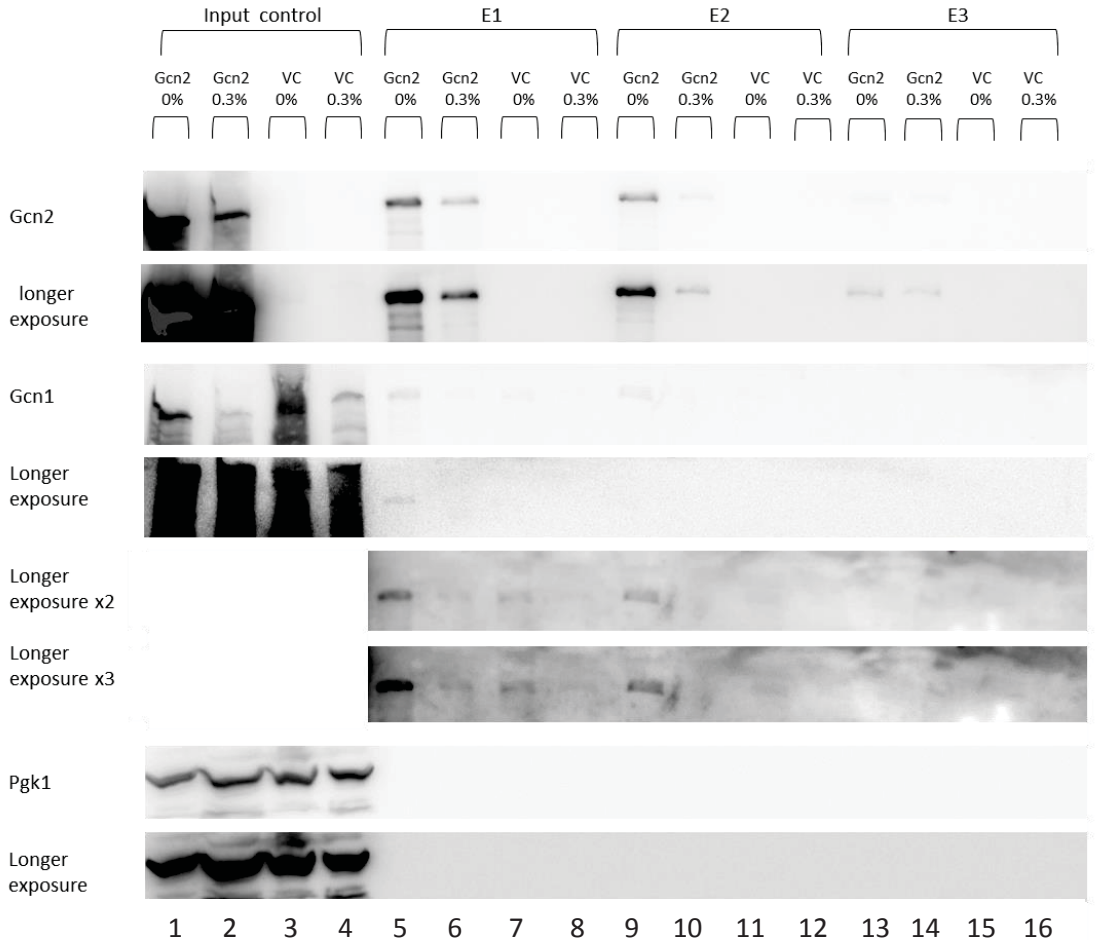


Figure 3.25 Large scale iMAC mediated purification of Gcn2. 24 Four 300ml cultures of his_6-Gcn2 expressing and vector control containing strains were grown to exponential phase. One set of each strain was treated with 0.3% formaldehyde. Cell extract was prepared using mechanical lysis. Four pull-down assays were performed in tandem. For this, 1000µg total protein of each sample was precleared and subjected to iMAC-mediated Gcn2 purification. Each of the four samples were joined and then Gcn2 was eluted from the beads using 200µl 250mM Imidazole. The samples were subjected to SDS-PAGE and immunoblotted using antibodies against Gcn2, Gcn1 and Pgk1.

Chapter 4

Discussion

4.1.0 The identification of potential Gcn2 binding proteins

General control non-derepressible 2 (Gcn2) is a key component of the general amino acid control (GAAC) pathway and its function in this pathway has been well characterised (Castilho et al., 2014). The canonical role of Gcn2 is to sense and respond to amino acid deplete conditions, enabling an organism to overcome amino acid deprivation. In addition to its well established role in the GAAC pathway, Gcn2 has been implicated as a key component in many additional aspects of cellular homeostasis including DNA damage checkpoint regulation (Menacho-Marquez, Perez-Valle, Arino, Gadea, & Murguia, 2007) and hypoxic stress response (Y. Liu et al., 2010). It is still not clear how Gcn2 executes the correct function at the correct time. Studies strongly suggest that this is accomplished by protein interactions that moderate Gcn2 activity (Castilho et al., 2014). The role of Gcn2 in the GAAC pathway has been extensively studied, however, the negative regulation of Gcn2 by eEF1A has only recently been recognised (Visweswaraiah et al., 2011). Gcn2 is also involved in a myriad of other cellular processes and pathways, which implies that there may be additional proteins that regulate Gcn2 activity for alternate functions. To support the supposition that not all Gcn2 regulatory proteins are known yet, large scale affinity purification studies have reported identification of many novel potential Gcn2 binding proteins (Breitkreutz et al., 2010; A.-C. Gavin et al., 2002; A. C. Gavin et al., 2006; Ho et al., 2002). Thus, identification of novel Gcn2 in vivo protein interaction partners would provide a more comprehensive understanding of Gcn2 regulatory mechanisms.

Five large scale protein-protein interaction screens have been published so far. In these studies, yeast strains were used where one protein at a time was tagged with an epitope tag to enable its purification and subsequent identification of bound proteins via mass spectrometry. These data sets from these five studies were examined to find proteins that potentially bind to, or are in complex with Gcn2, with the aim of constructing a Gcn2 interactome i.e. a comprehensive dataset of potential Gcn2 binding proteins, that could be analysed to identify prospective Gcn2 interaction partners for further investigation. The five large scale studies, performed by Ho et al 2002, Breitkreutz et al 2010, Krogan et al 2006, Gavin et al 2002 and Gavin et al 2006, all involved tagged protein purification, however the experimental conditions differed. There is a possibility that these differences biased the preservation of disparate protein interactions in each of the studies.

Ho et al 2002 and Breitkreutz et al 2010 utilised FLAG and HA tag epitopes which involve single step affinity purification of tagged proteins. FLAG and HA tags confer the advantage of being particularly small, the FLAG tag is eight amino acids in length (Hopp et al., 1988) and the HA tag is composed of 9 amino acids (Field et al., 1988). The small size of the tags make it unlikely that the tag will interfere with protein function or binding of interacting proteins to the tagged protein.

In these two studies, tagged bait proteins were overexpressed from a galactose inducible promotor. Overexpression of a protein can potentially preserve protein-protein interactions by mass action, thereby increasing the likelihood of driving transient interactions. This was of particular interest to our study, because protein-protein interactions for regulatory purposes, such as those likely to occur for Gcn2 regulation, tend to be weak or transient (Castilho et al., 2014).

The studies conducted by Krogan et al 2006, Gavin et al 2002 and Gavin et al 2006 all utilised TAP tagged proteins for purification, involving two affinity purification steps. The proteins were expressed from their endogenous promotors at native levels therefore no weak or transitory protein-protein interactions would be would be driven by mass action. The ability of proteins to bind to the bait may have also been influenced by the size of the TAP tag, which is 172 amino acids in length (Rigaut et al., 1999).

There were additional differences in experimental conditions between the studies that may have only been conducive for the preservation of some interactions, while being detrimental to others. For example, the cell lysate preparation methods varied between the five studies, and the concentrations of reagents used in the lysis buffers such as salts and detergents also differed, with the exception of the two studies by Gavin et al (2002 and 2006) (Table 4.1). These variations may affect protein interactions within or during the preparation of a cell lysate as certain bonds would be disrupted and others would be stabilised as a consequence of the materials and/or methods utilised in the studies (Nyborg & Peersen, 2004; Schreiber, 2002). For example, ionic interactions would be weakened by higher salt concentrations, while hydrophobic interactions would be more stabilised. The type of proteins bound non-specifically to an affinity matrix, through ionic or hydrophobic interactions, would also differ accordingly due to the buffer composition used during purification.

The preparation of samples for Mass Spectrometry also differed between the studies. In some of the studies, the samples were subjected to gel electrophoresis. The protein bands were then visualised by coomassie or silver staining, and the visible bands were excised and analysed, meaning that complexes that were not abundant in the sample, may have been undetected and therefore not analysed by Mass Spectrometry. Other studies performed analysis of the entire eluate. While this increases the change of detecting Gcn2 binding proteins in the sample that are lowly abundant, it also increases the possibility of detecting contaminant proteins that non-specifically bound to the affinity purification resin.

Table 4.1. Comparison of lysis buffer components used in large scale AP-MS studies

	Ho et al 2002	**Breitkreutz et al 2010**	**Krogan et al 2006**	**Gavin et al 2002**	**Gavin et al 2006**
Buffer	50mM HEPES pH 7.5	50mM HEPES pH 7.5	100mM HEPES-KOH pH 7.9	10mM K-HEPES pH 7.9	10mM K-HEPES pH 7.9
Salt	150mM NaCl	150mM NaCl	250mM KCl	10mM KCl	10mM KCl
Detergents	0.5% Triton X-100	-	-	-	-
Chelating agents	1mM EDTA	5mM EDTA	1mM EDTA	-	-
Reducing agents	0.5mM DTT	-	2.5mM DTT	0.5mM DTT	0.5mM DTT
Metal ions	10mM MgCl2	-	-	1.5mM MgCl2	1.5mM MgCl2
Protease inhibitors	benzamidine, leupeptin, aprotinin, AEBSF, pepstatin A.	1X complete protease inhibitors (Roche)	-	PMSF, benzamidine, leupeptin, pepstatin A, chymostatin, aprotinin	PMSF, benzamidine, leupeptin, pepstatin A, chymostatin, aprotinin
Phosphatase inhibitors	B-glycerophosphate, NaF	-	-	-	-

The published affinity purification studies were a valuable resource for identifying potential novel Gcn2 binding proteins, however the data needed to be carefully analysed to determine which candidate proteins were likely true Gcn2 binding partners. The disparities between the purification procedures used in the large scale screens effectively enabled us to narrow down the list of putative Gcn2 binding proteins by prioritising interactions that were found in two or more studies. It was reasoned that if

an interaction was found in two or more non-identical purification procedures then it was more likely to be genuine.

In order to identify potential Gcn2 binding partners, a raw dataset was generated which included all of the proteins from the five studies that were co-precipitated by tagged Gcn2 (bait) as prey, all of the tagged bait proteins that co-precipitated Gcn2 (prey) and all of the additional proteins that were co-precipitated as prey proteins alongside Gcn2 (prey) by these bait proteins. This dataset was compiled using the Microsoft excel programme. Two separate Gcn2 interactomes were then generated using this raw dataset, named the primary and secondary Gcn2 interactomes.

The primary Gcn2 interactome, contained all of the proteins that were co-precipitated with Gcn2 as bait or prey. Prey proteins that were co-precipitated in parallel with Gcn2 (prey) by bait proteins were omitted from the primary Gcn2 interactome because it is possible that the bait protein forms two independent complexes, one which includes Gcn2 and the other which includes the other prey protein. Therefore the primary Gcn2 interactome contained only the candidate proteins that we considered most likely to bind to, or form a complex that contains, Gcn2 (Figure 3.4).

The secondary Gcn2 interactome was compiled using only the prey proteins that were co-precipitated at the same time as Gcn2 (prey) by bait proteins, which had been omitted from the primary interactome. We reasoned that if Gcn2 and a prey were co-precipitated by two separate baits in two different studies then there is a possibility that Gcn2 and the prey protein may both be components of the same complex.

4.1.1 The interactome analysis did not identify all of the known Gcn2 binding proteins

Gcn2 is already known to interact with specific proteins. Accordingly, one would expect that these proteins would have been found in the published large scale protein-protein interaction screens as Gcn2 binding proteins. Thus, analysis of these studies in regards to these known Gcn2 binding partners provided us with an indication of how likely we were to identify novel Gcn2 binding partners using these studies.

It has been shown that Gcn2 directly contacts Gcn1 (Sattlegger & Hinnebusch, 2000a), and that Gcn1 contacts Gcn20 (Vazquez de Aldana et al., 1995), suggesting that these three proteins form a complex. Gcn1 was not identified in the primary interactome, as it did not co-precipitate Gcn2 as bait or prey. Gcn1 was found to co-precipitate in parallel with Gcn2 by five different baits, however these were all found in the same study (Krogan et al., 2006) and therefore Gcn1 was also not included in the secondary interactome. The reason for this may be because the interaction between Gcn2 and Gcn1 is weak and therefore not robust enough to be preserved during the co-purification process. Gcn20 was altogether absent from the raw data set.

Known Gcn2 binding protein Heat shock protein 90 (Hsp90) exists in two isoforms coded for by the genes Hsp82 and Hsc82 (Donzé & Picard, 1999). Hsp90 was also not identified in the primary or secondary interactomes. However there were seven cases in which a bait protein co-precipitated either Hsp82 or Hsc82 and Gcn2 in parallel, however these were all found in only one study (Krogan et al., 2006).

Together, this suggests that proteins found in only one study to co-purify alongside Gcn2 may be true interaction partners. However, due to time constraints, we chose to omit such proteins, and instead prioritise interactions that were observed in two or more studies, as these were the most likely to be authentic interaction partners.

The predominant absence of these known Gcn2 binding partners in the five large scale studies, indicates that for a protein to co-precipitate with Gcn2 and therefore be identified as a potential binding partner, a strong interaction may be required. Thus it can be presumed that the proteins that did co-precipitate with Gcn2 in these studies, in particular those found in more than one study, potentially form a robust bond with Gcn2, more robust than the Gcn1-Gcn2 interaction for example. However, it is likely that many proteins that regulate Gcn2 function, do so through weak or transient interactions, and these will likely not have been identified in this analysis.

Although some known Gcn2 binding partners were not identified in the interactome analysis, eukaryotic translation Elongation Factor 1 A (eEF1A), encoded by the gene *TEF2*, co-precipitated with Gcn2 as prey and therefore featured in the primary interactome (A. C. Gavin et al., 2006). eEF1A has previously been determined to bind to, and repress Gcn2 activation under replete conditions, (Visweswaraiah et al., 2011). The presence of eEF1A in the primary interactome indicates that the bond that it forms

with Gcn2 may be stronger than the bond that Gcn2 forms with the other known interaction partners that do not feature in the primary or secondary interactomes, at least under the particular experimental conditions used in this affinity purification study.

It shall be noted that the large scale affinity purification studies were all performed using cell cultures that were grown under nutrient-replete conditions. Regulatory interactions, such as those likely to occur between Gcn2 and its interaction partners, may occur only upon requirement for Gcn2 activation, during amino acid starvation. This means that the replete experimental conditions may bias the co-precipitation partner identification towards Gcn2 negative regulators, and potentially fail to capture Gcn2 positive regulatory interactions.

4.1.2 Proteins identified in the primary Gcn2 interactome analysis as both bait and prey

In the primary Gcn2 interactome we identified two proteins, Hrr25 and Cdc48, which co-precipitated with Gcn2 as both prey and bait in two separate studies, each utilising different affinity purification epitopes.

In the publication by Breitkreutz et al both HA and FLAG tagged bait proteins were used for affinity purification, Hrr25 (bait) co-precipitated Gcn2, and vice versa using HA tagged bait proteins, and Gcn2 (bait) co-precipitated Hrr25 in the FLAG tag mediated purification. In addition to this, Hrr25 (bait) co-precipitated Gcn2 in the TAP tag study conducted by in Gavin et al (2006). Considering that Gcn2 and Hrr25 were found to co-precipitate in several independent studies, this strongly suggests that Hrr25 is a Gcn2 binding protein.

Like Gcn2, Hrr25 is a serine/threonine-protein kinase that is implicated in the regulation of an extensive array of cellular processes. One of the substrates of Hrr25 phosphorylation is Ltv1, which is crucial for ribosomal biogenesis, export and subunit assembly (Ghalei et al., 2015; Ray et al., 2008). Interestingly, Ltv1 was also identified in our secondary Gcn2 interactome, suggesting that if Hrr25 does regulate Gcn2, it may do so in parallel to or via Ltv1. Other processes that Hrr25 is associated with, include vesicular trafficking (Lord et al., 2011) and cytoplasm-to-vacuole (CVT) pathway regulation (Pfaffenwimmer et al., 2014). Specific mutations in the Hrr25 gene result in

cellular inability to repair double stranded breaks in the DNA, signifying that Hrr25 is also important for DNA repair (Ghalei et al., 2015).

The frequency in which Hrr25 appears in our interactome analysis strongly suggests that Hrr25 is an authentic Gcn2 binding protein and further investigations should be conducted to validate this interaction.

Cdc48 was identified as a prey protein that co-purified with FLAG tagged Gcn2 (bait) in the study conducted by Ho et al (2002). In the study that utilised the TAP tag, conducted by Krogan et al (2006), Cdc48 (bait) co-precipitated Gcn2 as a prey. This suggests that Cdc48 and Gcn2 are binding partners, or in the same complex.

Cdc48 is an ATPase that is essential for cell viability. Cdc48 activity has been recorded in response to various forms of cellular stress such as DNA damage, UV irradiation and nutrient stress (Ossareh-Nazari et al., 2010; Verma, Oania, Fang, Smith, & Deshaies, 2011), however the roles of Cdc48 in cell cycle regulation and endoplasmic reticulum associated protein degradation (ERAD) have been the most extensively characterised.

During endoplasmic reticulum associated protein degradation CDC48, along with its cofactors Ufd1 and Npl4, identifies misfolded proteins and draws them out of the endoplasmic reticulum. The Cdc48-Ufd1-Npl4 complex then delivers the misfolded proteins to the proteasome for degradation (Jarosch et al., 2002; Raasi & Wolf, 2007; Y. Ye, Meyer, & Rapoport, 2001). Ufd1 and Np14 were not identified in our interactome analysis.

Interestingly Ubp3 and Bre5 which are also known Cdc48 interaction partners were identified in the primary interactome as potential Gcn2 binding proteins. These proteins are involved in the ubiquitination process. Ubiquitin is a small protein that is conserved among eukaryotes and abundant within the cell (Hershko, 1998). Ubiquitination and de-ubiquitination of proteins govern many processes required for cellular regulation and homeostasis. Mono- or poly-ubiquitination of a substrate functions as a cellular ‘signal’, the inference of this signal is determined by the substrate itself and the extent of the ubiquitination. Specific poly-ubiquitination of a protein acts as a “marker” to target proteins for degradation by the 26S proteasome (Chau et al., 1989; Finley et al., 1994).

Cleavage of the ubiquitin modification is performed by deubiquitinating enzymes (Varshavsky, 1997; Wilkinson, 1997). Ubiquitin-specific protease 3 (Ubp3) is a

deubiquitinating enzyme which functions in a complex with Bre5 (Baker, Tobias, & Varshavsky, 1992; Cohen, Stutz, Belgareh, Haguenauer-Tsapis, & Dargemont, 2003). The Ubp3-Bre5 deubiquitinase complex has been implicated in regulatory pathways which include controlled mitochondrial degradation (mitophagy) (Müller et al.) and its activity is essential for promoting starvation induced ribophagy of the 60S ribosome (Kraft, Deplazes, Sohrmann, & Peter, 2008).

Ribophagy is the selective degradation of mature ribosomes mediated by the ubiquitin pathway (Warner, 1999). During periods of nutrient stress not only is the synthesis of proteins repressed but the 'machinery' for protein synthesis is also downregulated via riboautophagy. Interaction of Cdc48 with the Ubp3-Bre5 complex is essential for Ubp3-Bre5 mediated ribophagy efficiency (Ossareh-Nazari et al., 2010).

The primary Gcn2 interactome was composed of proteins, from the raw dataset, that had the highest probability of being true Gcn2 binding partners. The appearance of both Cdc48 and specific Cdc48 co-factors in the primary Gcn2 interactome strongly indicates that these proteins form a complex that associates with or includes Gcn2. The Cdc48, Ubp3, Bre5 complex is already known to function in response to nutrient starvation stress by promoting ribophagy. Taken together, these observations indicate that Cdc48 should be prioritised for examination as a Gcn2 interaction partner.

Cdc48 featured further in our analysis, as a constituent of another potential complex comprising Cdc48, Shp1 and Gcn2 (discussed further in the next chapter).

Hrr25 and Cdc48 are both essential proteins, therefore a deletion of either gene would render the strain inviable. These priority candidate proteins could therefore not be included in our screen of single gene deletion mutants for SM sensitivity. Additionally the *CDC48* was not available in the plasmid overexpression library. Hrr25 was screened for SM sensitivity upon overexpression and did not display a SM^s phenotype indicating that it does not function in Gcn2 negative regulation. To test whether these proteins potentially function in positive regulation of Gcn2, semi-quantitative growth assays could be performed using strains from the Decreased Abundance by mRNA Perturbation (DAmP) collection (Breslow et al., 2008). Each strain in this library expresses a specific mRNA that is disrupted in its 3' untranslated region (UTR) by an antibiotic resistance cassette (kanamycin). This disruption destabilizes the mRNA leading to 4-10 fold reduction in mRNA translation, the levels of corresponding protein

within the cell are consequently reduced. If the protein functions in positive Gcn2 regulation, a decrease in the cellular protein levels may impede Gcn2 activation upon amino acid starvation, resulting in a SM^s phenotype.

4.1.3 Identification of potential complexes that contain Gcn2

The primary and secondary Gcn2 interactomes were generated to identify individual proteins that potentially bind to, or are in a complex with, Gcn2. To further elucidate the protein composition of complexes that contain Gcn2, we examined the primary and secondary interactomes for the occurrence of proteins that were repeatedly co-precipitated in parallel. Three potential complexes were identified.

The first distinct complex included Gcn2, Cdc48 and Shp1 (Figure 3.5). Cdc48 had already been identified as a protein of interest as it frequently co-precipitated with Gcn2 (see previous chapter). Studies have determined that specialised roles performed by Cdc48 are largely dependent on the complex that it resides in (Baek et al., 2013). The formation of a complex composed of Cdc48 and Shp1 has been previously reported (Braun et al., 2002; Schuberth et al., 2004). The Cdc48, Shp1 complex functions to positively regulate Glc7, the catalytic subunit of type 1 protein phosphatase (PP1). During the analysis of our compiled interactome data the presence of Glc7 in the secondary interactome was noted. The suggestion that Glc7 may be in complex with Gcn2 is interesting as Glc7 is the eIF2α phosphatase, and thus counteracts Gcn2. This raises the intriguing possibility that Cdc48, Shp1, Glc7 and Gcn2 could be components of the same complex. (Wek, Cannon, Dever, & Hinnebusch, 1992).

It would be reasonable to postulate that Glc7 may function in close proximity to Gcn2, as eIF2α would require rapid de-phosphorylation when amino acids are no longer limited to efficiently down-regulate the stress response pathway. Further investigations are required to determine if Cdc48 regulates Gcn2, and if so, whether Glc7 is required for this regulation.

The second potential complex was composed of Gcn2, Gcd11, Tba1 and Hca4 (Figure 3.6). Gcd11, also referred to as Sui4 is the γ subunit of eIF2 (Harashima & Hinnebusch, 1986b). Tba1 is a transcription factor, one of its roles is to binds GCN4 to repress transcription of ribosomal protein genes (Joo, Kim, Kang, Yu, & Kim, 2011). The last

protein Hca4 is an RNA helicase that may have a role in 18s ribosome synthesis (Liang, Clark, & Fournier, 1997). An interaction between Gcn2 and Gcd11 is plausible, as Gcd11 is a subunit of the Gcn2 phosphorylation substrate. Considering the known functions of Tba1 and Hca4, transcription factor and a RNA helicase respectively, it seems improbable that they would associate with Gcn2. However it cannot be discounted, as this interaction may occur via component shuttling into and out of the nucleus to regulate Gcn2 i.e. as part of a regulatory feedback loop.

The third complex comprised Gcn2, Pph3 and Psy2 (Figure3.7). Pph3 and Psy2 are both subunits of the protein phosphatase (PP4) complex (B. M. O'Neill et al., 2007). One of the de-phosphorylation targets for this complex is Rad53 which is a DNA damage checkpoint regulatory kinase. Recovery from DNA damage is dependent on the de-phosphorylation of this target (S. Chen, Smolka, & Zhou, 2007). Gcn2 has also been implicated in cell cycle regulation in response to DNA damage (Grallert & Boye, 2007) suggesting that this phosphatase complex may interact with Gcn2 to elicit its activation in response to DNA damage. Although Gcn2 was co-precipitated as prey by each of these proteins neither were co-precipitated by Gcn2, therefore, although it is possible that Gcn2 forms a complex with Pph3-Psy2, it is also possible that this complex interacts with an alternate protein that is in a complex with Gcn2.

4.1.4 Identification of new regulators that promote Gcn2 activity

The Gcn2-interactome analysis identified novel potential Gcn2 binding proteins. The next logical step would be to conduct experiments to determine whether these novel proteins are truly in complex with Gcn2. However, the amount of proteins to be tested were too numerous and would have been out of the scope of this thesis. Since not all of the putative Gcn2 binding partners necessarily regulate Gcn2, and because our main interest was to find novel Gcn2 regulators, the next objective was to screen the potential novel Gcn2-binding partners for those that promote Gcn2 activity. For this we used single gene deletion strains that each lacked one of the 60 novel potential Gcn2 binding proteins, to identify strains that were unable to overcome starvation for branched chain amino acids, elicited by the drug Sulfometuron methyl (SM). This SM^s phenotype was indicative of the missing protein being required to positively regulate Gcn2.

Of the 60 deletion strains, 20 showed sensitivity to SM and three of the strains had decreased eIF2α-P levels under starvation conditions when compared to the wild type strain: *chs5Δ*, *ncl1Δ* and *sse1Δ*. This suggested that each of these three proteins are required for promoting Gcn2 activation, the distinct functions of all of the three proteins are known.

Chs5 is involved in vesicular transport of proteins (Cid et al., 1995). It is possible that its absence affects the distribution of proteins and the composition of protein complexes such as complexes containing Gcn2, thereby impairing Gcn2 activation.

Ncl1 is a tRNA methyltransferase activated under specific cellular stress conditions (Motorin & Grosjean, 1999; Wu, Brockenbrough, Paddy, & Aris, 1998). In response to hydrogen peroxide, Ncl1 modifies $tRNA^{leu}$ (CAA) resulting in increased translation of genes with high levels of TTG codons (Chan et al., 2012). Gcn2 is also activated under conditions of oxidative stress and increased Gcn4 translation is part of the cellular response for overcoming the oxidative stress (Mascarenhas et al., 2008).

Sse1 is a member of the Hsp70 group of proteins that forms part of a greater Hsp90 chaperone complex (Mukai et al., 1993). The Hsp70/Hsp90 complex is part of a mechanism that promotes cellular homeostasis by monitoring and processing newly synthesized proteins. Hsp90 is a chaperone required for assisting Gcn2 folding and subsequent maturation of newly translated Gcn2. It was proposed that Hsp90 remains bound to mature Gcn2, and that this interaction is released upon binding of tRNA to the HisRS domain of Gcn2 (Donzé & Picard, 1999). Since Hsp90 (Hsp82) is in complex with Gcn2 (X. D. Liu, Morano, & Thiele, 1999), this supports the idea that Sse1 may be part of this complex and could participate in Hsp90 mediated Gcn2 maturation or regulation.

The decreased eIF2α-P levels found in the *chs5Δ, ncl1Δ* and *sse1Δ* strains under amino acid starvation, supported the idea that the gene products are involved in the positive regulation of Gcn2 activation or activity. It should be noted that the strains used were sourced from a collection of unverified strains. Therefore, the next stage would be to perform complementation assays to validate that the observed phenotype is truly due to the deleted gene and not the result of an ectopic mutation. If re-introduction of the gene does not revert the SMs phenotype, then this phenotype results from an ectopic mutation rather than the loss of the investigated protein. However if the wild type

phenotype is restored then this would support the idea that the investigated protein is a Gcn2 activator, and its mechanism of Gcn2 regulation should be further examined. The strains for this complementation procedure were transformed successfully, these experiments are ongoing.

Seven of the SM^s strains did not show any change in eIF2-P levels when compared to the wildtype: *ubp15Δ, ssa1Δ, ssa2Δ, ltv1Δ, hsl1Δ, shp1Δ* and *tah1Δ*. This suggests that the corresponding proteins are not required for promoting Gcn2 activation. However this inference is not conclusive, as it cannot be discounted that the proteins regulate Gcn2 under a separate set of conditions to those performed in our experimental procedure.

Interestingly five of the SM sensitive strains were observed to have increased levels of eIF2α-P upon starvation for amino acids when compared to the wild type strain; *ssb1Δ, ysp1Δ, psy2Δ, nhp6aΔ* and *ubp3Δ*. And two of these strains displayed a significant increase in eIF2α-P, *ubp3Δ* and *ssb1Δ*.

Increased eIF2α-P levels would suggest that the corresponding proteins are not required for promoting Gcn2 function. Instead, these proteins may impair a step in the GAAC signalling pathway down stream of Gcn2, in which case Gcn2 may attempt to compensate for this defect by hyper-phosphorylating eIF2α. Further testing may provide insight into whether these candidate proteins influence the GAAC pathway downstream of Gcn2. For example constitutively high Gcn4 expression should restore the wild type SM^s phenotype and levels of eIF2α-P, if the defect in GAAC was due to impaired induction of Gcn4 translation.

SSB1 codes for a chaperone protein that is part of a subfamily of Hsp70 proteins which is implicated in protein folding (Werner-Washburne, Stone, & Craig, 1987). As discussed above, Gcn2 is known to interact with another member of the HSP group Hsp90 (Donzé & Picard, 1999). Additionally, another large scale affinity purification study identified Ssb1 as a co-purification partner of known Gcn2 interaction partner Gcn1 (Babu et al., 2012). Since Gcn1 binds to Gcn2, this would support the idea that Ssb1 may be in complex with Gcn2. It is therefore possible that Ssb1 has a role in counteracting the negative regulatory function of Hsp90. Further investigation into the role of Ssb1 in the GAAC pathway may be warranted.

Ubp3 featured previously in our analysis as it was identified as a Cdc48 co-factor required for efficient ribophagy under starvation conditions (chapter 3.1.4). One of the additional targets for Ubp3-Bre5 deubiquitination is TATA-binding protein 1 (Tbp1). Tbp1 is a transcription factor that induces transcription of *GCN4*. Deletion of *UBP3* results in increased poly-ubiquitination and degradation of Tbp1, a consequent reduction in the transcription of *GCN4* (Chew, Siew, Xiao, & Lehming, 2010), and decreased cellular Gcn4 mRNA levels. The increased eIF2-P observed in the ubp3Δ strain under amino acid starvation conditions, would most likely be due to an increase in Gcn2 activity in a futile attempt to enhance translation of Gcn4 mRNA, to overcome amino acid starvation.

4.1.5 Screening for potential negative regulators of Gcn2

The aim of section 3.2.1 was to identify proteins that function in positive regulation of Gcn2, by conducting growth assays using single gene deletion mutants. This approach however is not suitable for identifying proteins that inhibit the activity of Gcn2. To identify potential Gcn2 negative regulators, we overexpressed the candidate proteins in the cells and screened for SM sensitivity. The reason for this was that if a protein functions in Gcn2 inhibition, then higher expression of that protein may result in stronger Gcn2 inhibition and an impaired ability to activate Gcn2 upon starvation. This would result in a SM sensitive phenotype.

Of the 12 proteins screened for impaired Gcn2 activity upon overexpression, two of the strains displayed a SM^s phenotype. These were strains overexpressing Tir4 and Npr1.

Interestingly, the Tir4 overexpression strain had significantly increased levels of eIF2α-P under replete conditions, while eIF2α-P levels under amino acid starvation were similar to those observed in the wild type strain. This indicated that Tir4 is not required for promoting Gcn2 activation during amino acid starvation. Under replete conditions Tir4 overexpression appeared to positively regulate Gcn2, leading to increased Gcn2 basal activity. If Tir4 is a Gcn2 activator, then a Tir4 deletion strain should exhibit a SM^s phenotype, but this was not the case, at least in our studies. This may be because Tir4 is preferentially expressed during hypoxia and cold shock (Abramova, Sertil, Mehta, & Lowry, 2001), and the low Tir4 levels under non-stressed conditions may not be enough to promote Gcn2 function. In line with this idea, Gcn2 is known to be

activated under hypoxic conditions (Y. Liu et al., 2010). If Tir4 were required to promote Gcn2 activation only under hypoxic stress or cold shock, over expression of Tir4 may mimic these stress conditions accounting for the increased levels of eIF2α-P. However, in that case it would be expected that a strain would display a SM resistant phenotype, and we found that the overexpression strain was SM^s. It may be that in our strain, TIR4 is expressed at higher levels than it would be under hypoxic conditions. If Tir4 does positively regulate Gcn2, than this may result in Gcn2 hyper activation, and the consequential arrest of global protein translation. The cells would therefore be unable to translate the proteins that are essential for amino acid biosynthesis to overcome the starvation, resulting in a SM^s phenotype.

Nitrogen permease reactivator (Npr1) is a serine/threonine protein kinase that functions in the regulation of membrane amino acid transporters. Regulation of Npr1 is exerted by the Target Of Rapamycin (TOR) pathway. Like the GAAC pathway, the TOR pathway is activated in response to specific cellular stress. One of the stress conditions that the TOR pathway responds to is nitrogen insufficiency. Under conditions of nitrogen starvation Npr1 undergoes phosphorylation at multiple sites by the Tor1 subunit of the TOR complex, and this phosphorylation inhibits Npr1 activity (Gander et al., 2008). Interestingly, known TOR pathway components Tor1, Tor2 and Snf1 were also present on our secondary Gcn2 interactome dataset.

Npr1 had been previously validated as a Gcn2 binding partner (Breitkreutz et al., 2010). The Npr1 overexpression strain was observed to have increased eIF2α-P levels under amino acid starvation conditions, when compared to the wild type strain (Figure 3.14). This was unanticipated as it would be expected that a SM^s phenotype in a strain overexpressing a protein would correlate with decreased eIF2α-P levels, resulting from increased Gcn2 inhibition and therefore decreased Gcn2 activity.

The increased eIF2α-P levels indicated that overexpression of Npr1 may induce Gcn2 hyperactivity, which would suggest that Npr1 is a positive regulator of Gcn2. However *npr1Δ* strains did not display SM^s in our studies, which would suggest that Npr1 is not required for Gcn2 activation. Npr1 has a paralog named Prr2. Further investigations should be conducted to analyse whether Prr2 can functionally compensate for a deletion of Npr1. In this case, double deletion of *NPR1* and *PRR2* should render the cells SM^s.

If Npr1 is in fact a Gcn2 activator, and overexpression of Npr1 results in hyper-activation of Gcn2, unrestrained levels of eIF2α phosphorylation may result in the total cessation of global protein synthesis. The inability to synthesise any proteins upon amino acid starvation, would affect the essential cellular processes that rely on *de novo* protein synthesis. This would result in the severe growth defect on SM that we observed.

Taken together, analysis of the available data from five large scale published affinity purification studies revealed 135 proteins that potentially bind Gcn2 or form complexes that include Gcn2. Five novel potential Gcn2 regulating proteins were identified: Sse1, Chs5, Ncl1, Tir4 and Npr1. Although four of these proteins still need to be validated as Gcn2 binding proteins, and the Gcn2 regulatory function of all four proteins requires verification, these results positively indicate that, as we predicted, there are still Gcn2 regulating proteins that remain unidentified.

4.2 Establishment of a his_6 tag mediated Gcn2 affinity purification procedure.

Analysis of the published affinity purification studies performed in chapter 3.1 revealed a failure to identify many of the known Gcn2 binding partners in these studies, strongly suggesting that other novel Gcn2 binding partners were also not identified. The experimental conditions used in the large scale affinity purification studies may not have been optimal for the maintenance of Gcn2-protein interactions. Optimization of the experimental procedure was required to specifically identify proteins in complex with Gcn2. Therefore we wanted to identify Gcn2 affinity purification conditions that would be specifically conducive for the maintenance of bonds between Gcn2 and its interaction partners. Such an approach would be required to create a more comprehensive Gcn2 interactome.

To perform Gcn2 affinity purification while maintaining Gcn2-binding partner interactions, we first considered the levels at which Gcn2 should be expressed. Because Gcn2 interactions may be weak and transitory, as observed with the Gcn1-Gcn2 interaction (Garcia-Barrio, Dong, Ufano, & Hinnebusch, 2000), we decided that these interactions could be driven and preserved more effectively by overexpressing Gcn2. We therefore used a Gcn2 deletion strain that expressed plasmid-borne Gcn2 from a strong galactose inducible promotor. This plasmid had been used previously and was known to be functional (J. Dong et al., 2000). Experiments conducted by us showed that Gcn2 expressed using this plasmid resulted in intracellular Gcn2 levels that were approximately 20 fold higher than native levels of Gcn2.

To specifically conserve Gcn2-protein interactions throughout the purification process we used a low salt buffer (Garcia-Barrio et al., 2000) with 50mM KCl, since this had been shown previously to be necessary to preserve Gcn1-Gcn2 interactions. We also considered the usage of formaldehyde to further stabilise the weak and transient interactions (Markham, Bai, & Schmitt-Ulms, 2007). Although there are many crosslinking agents available, formaldehyde treatment was chosen since it has already been used successfully during Gcn1-affinity purification to stabilise protein-protein interactions with Gcn1, including the interaction of Gcn1 with Gcn2 (Shanmugam and Sattlegger unpublished). One of the benefits of using formaldehyde is that it is a small molecule which readily permeates the yeast cell wall and membrane, treatment is therefore possible on intact cells before cell breakage commences. Protein crosslinking

occurs almost instantaneously providing a 'snap-shot' of the interactions that are occurring within the cell at the time of treatment (Sutherland, Toews, & Kast, 2008) Additionally, only proteins that are in close association can be crosslinked since formaldehyde is a very small molecule of 2.3-2.7Å, which reduces the possibility of crosslinking proteins that are just in close proximity to each other (Sutherland et al., 2008).

Gcn2 purification was to be conducted via an N-terminal his_6 tag, using resin that contained a bivalent cation Ni^{2+}. In order to remove proteins un-specifically attached to the affinity resin, before elution we washed the resin with buffer that contained 30mM imidazole. Yeast naturally has proteins containing poly histidine stretches that may associate with the Ni^{2+} of the resin, and the imidazole aids in removing such interactions by competing for binding to the Ni^{2+}.

The Gcn2 affinity purification procedure was optimised successfully. Gcn1 was found to co-precipitate with Gcn2 without detectable contamination by Pgk1. Pgk1 is an abundant housekeeping protein, with each cell containing over 150,00 molecules (Chong et al., 2015), despite this high abundance it was not found to co-elute with Gcn2. Although Pgk1 was used as an indicator for contamination, we also performed the purification using a control strain that contained the vector alone, and therefore no Gcn2. Contaminant proteins that bind non-specifically to the affinity purification resin could be identified in eluates from this strain by Mass Spectrometry.

The next step was to identify the novel Gcn2 binding partners via Mass Spectrometry. First, only samples not treated with formaldehyde were analysed, since the amount of co-purified Gcn1 was greater in these samples when compared to the formaldehyde treated samples. Unfortunately, no protein, not even Gcn2 was identified in the samples. This is likely because the precipitate did not contain enough proteins for conclusive identification via Mass Spectrometry. Gcn2 as well as Gcn1 were detected in the affinity purification sample via western blotting, but not in Mass Spectrometry, possibly because the antibody against Gcn2 is very sensitive.

Nevertheless, the data strongly suggests that the affinity purification of Gcn2 and its binding partners, was successfully established. The next step is to repeat the affinity purification procedure but in larger scale to produce a more concentrated sample, for subsequent Mass Spectrometric analysis. This was out of the scope of this thesis.

Performing the interactome analysis using optimised conditions suitable for Gcn2 complexes will be crucial for generating a comprehensive Gcn2 interactome. Identification of Gcn2 containing complexes and subsequent analysis to identify Gcn2 regulating proteins is an important step in developing a greater understanding of the complex mechanisms of Gcn2 activity. Since the protein-protein interactions are dynamic and may depend on the cellular conditions, it will be interesting to conduct this interactome analysis under various conditions, such as replete and starved conditions.

4.3 Conclusion and future directions

The aim of this research was to identify novel Gcn2 regulators from the pool of potential Gcn2 interaction partners to further elucidate the multifaceted mechanism of Gcn2 regulation. In this study, through in depth analysis of five large scale affinity purification studies, new potential Gcn2 binding partners have been recognised. Of those, Sse1, Chs5, Ncl1, Tir4 and Npr1 were identified as potential novel Gcn2 regulators. The experimental conditions for identifying Gcn2 binding partners were not optimal in the high throughput studies. Therefore, an affinity purification procedure was successfully optimized that will enable a more comprehensive identification of Gcn2 binding proteins. This work has helped to further unveil members of the complex Gcn2 regulatory network, and has brought us a step closer to achieving a comprehensive understanding of Gcn2 regulation.

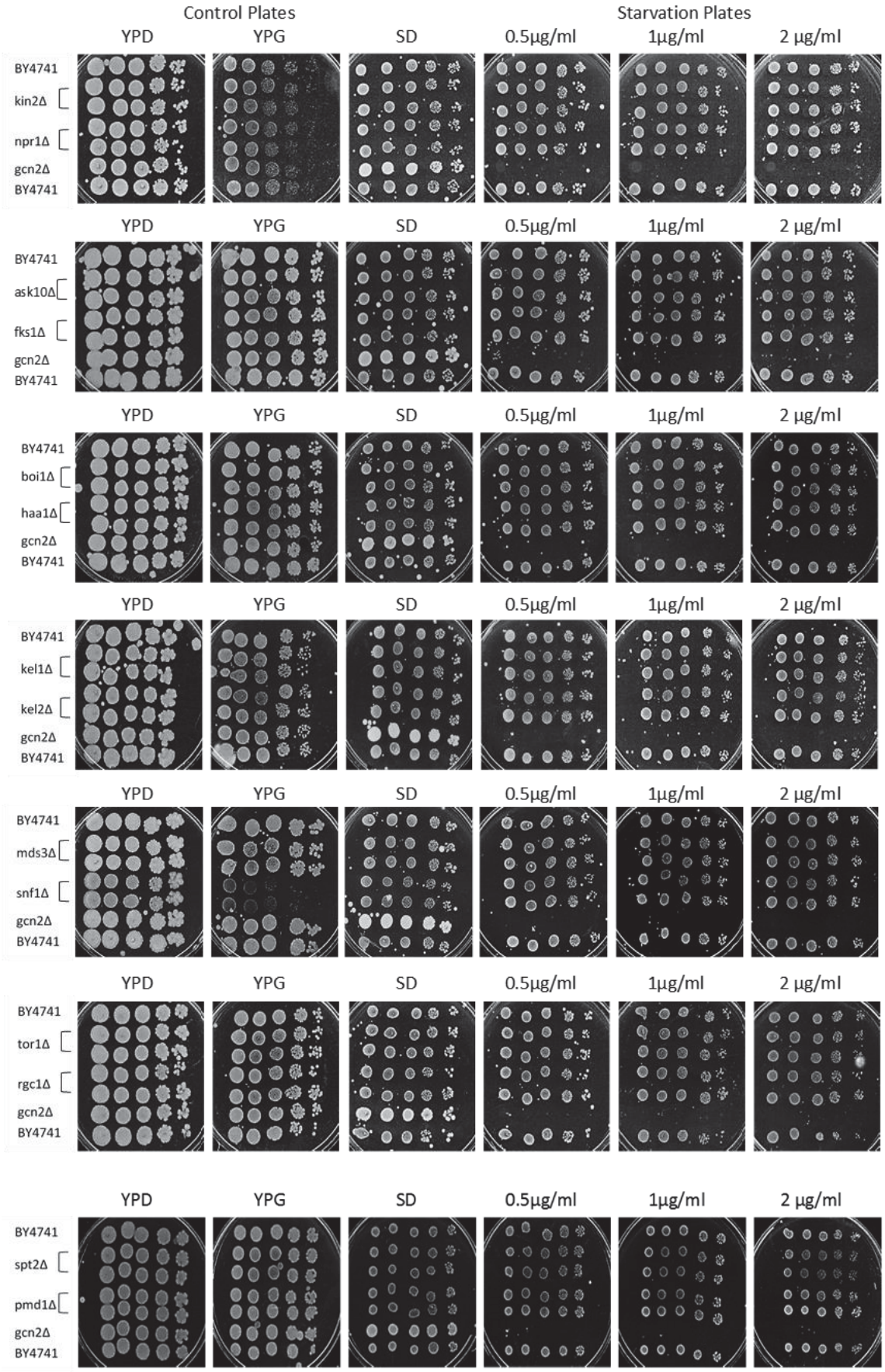
The novel Gcn2 binding partners found, still need to be validated as true Gcn2 binding partners. Our interactome analyses identified 135 potential binding partners, however this list is far from complete. In this thesis, the focus was on using a genetic approach, to screen the potential binding partners for those that promote Gcn2 activation, which identified Sse1, Chs5, Ncl1, Tir4 and Npr1 as potential Gcn2 activators. A screen for those that inhibit Gcn2 has been started, but due to the time constraints, more of the candidate proteins still need to be tested. Nevertheless, this study showed that the genetic approach is a feasible way to screen a large number of Gcn2 binding proteins for those that modulate Gcn2 activity, and thus to prioritise potential candidates for further in depth analysis.

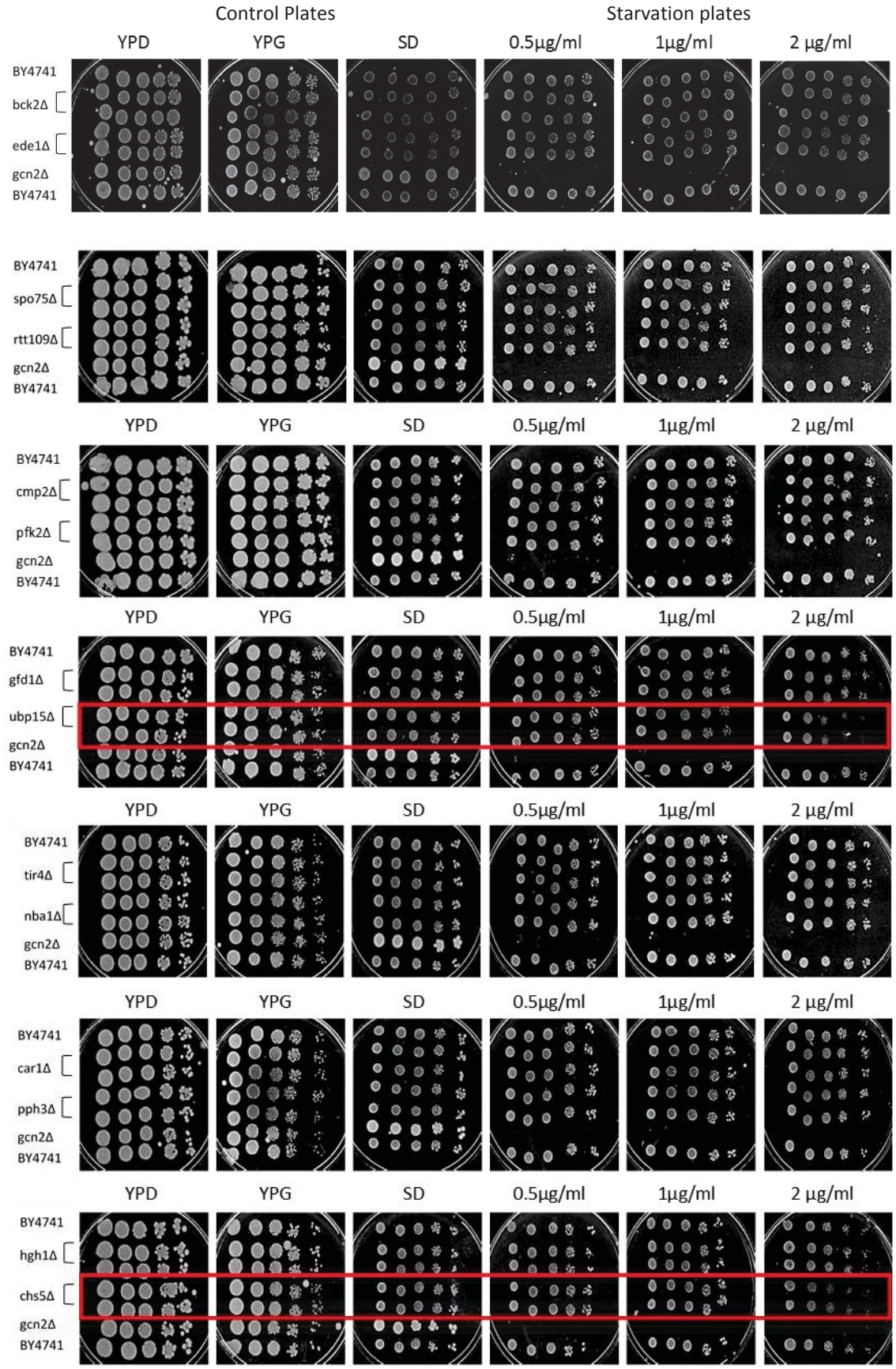
Now that a Gcn2 affinity purification procedure has been established, that preserves weak and transient interactions, it should be performed on a large scale, to more comprehensively identify new Gcn2 binding partners.

Gcn2 is a promising therapeutic target for the treatment of a wide range of diseases. Because the mechanisms of Gcn2 regulation are highly conserved (Castilho et al., 2014), research into Gcn2 activation and activity can be efficiently conducted using yeast. Understanding the mechanisms of Gcn2 regulation, through identification of proteins that modulate Gcn2 activity is a crucial step towards determining how Gcn2 activity can be manipulated for therapeutic purposes.

Appendix

Appendix 1 Results of SM^{s} Screen performed on single gene deletion mutants

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Starvation plates
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YPG
SD
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1µg/ml
2 µg/ml
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bck2Δ
ede1Δ
gcn2Δ
BY4741
BY4741
spo75Δ
rtt109Δ
gcn2Δ
BY4741
BY4741
cmp2Δ
pfk2Δ
gcn2Δ
BY4741
BY4741
gfd1Δ
ubp15Δ
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BY4741
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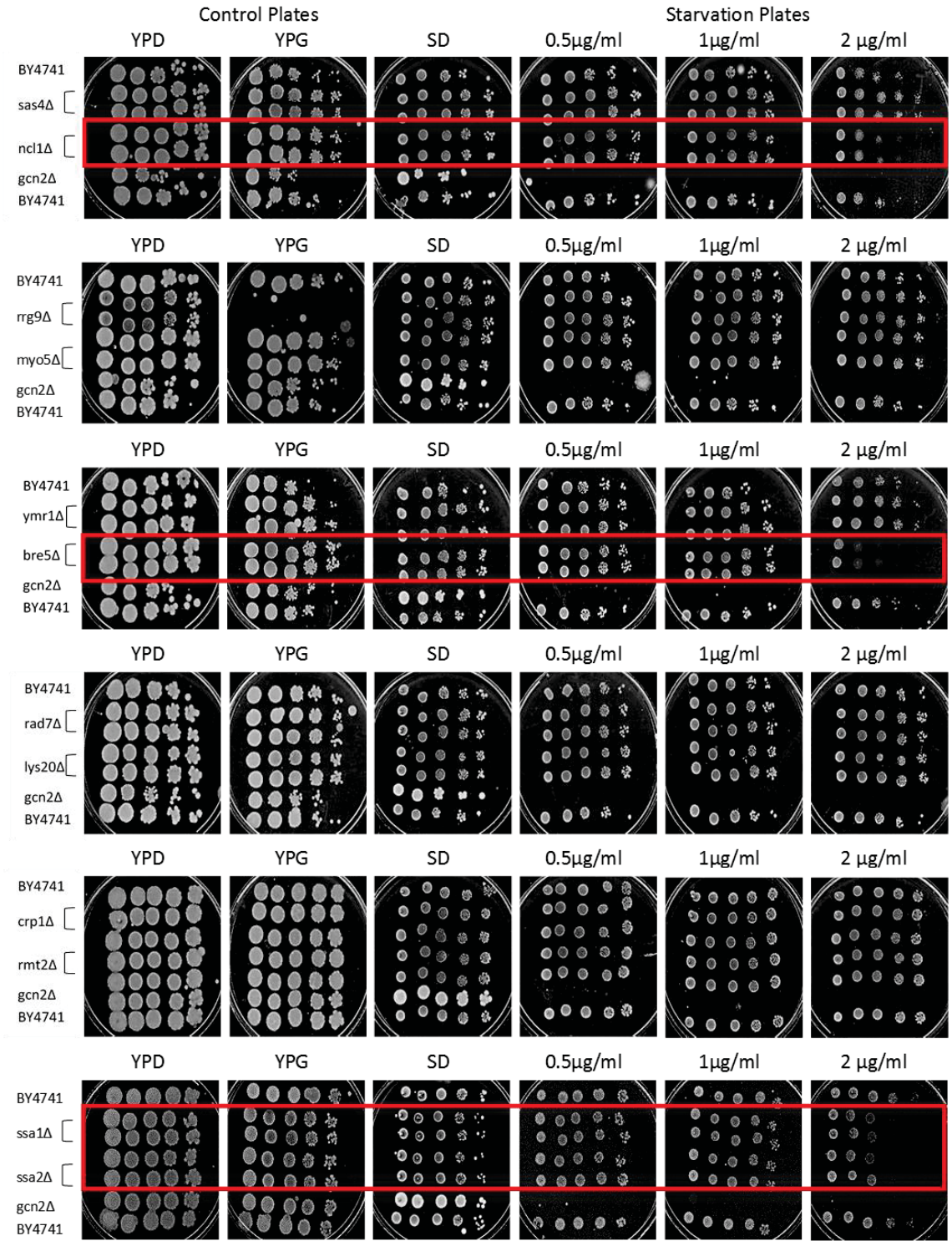
gcn2Δ
BY4741


Control Plates
Starvation Plates
YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
sas4Δ
ncl1Δ
gcn2Δ
BY4741
BY4741
rrg9Δ
myo5Δ
gcn2Δ
BY4741
BY4741
ymr1Δ
bre5Δ
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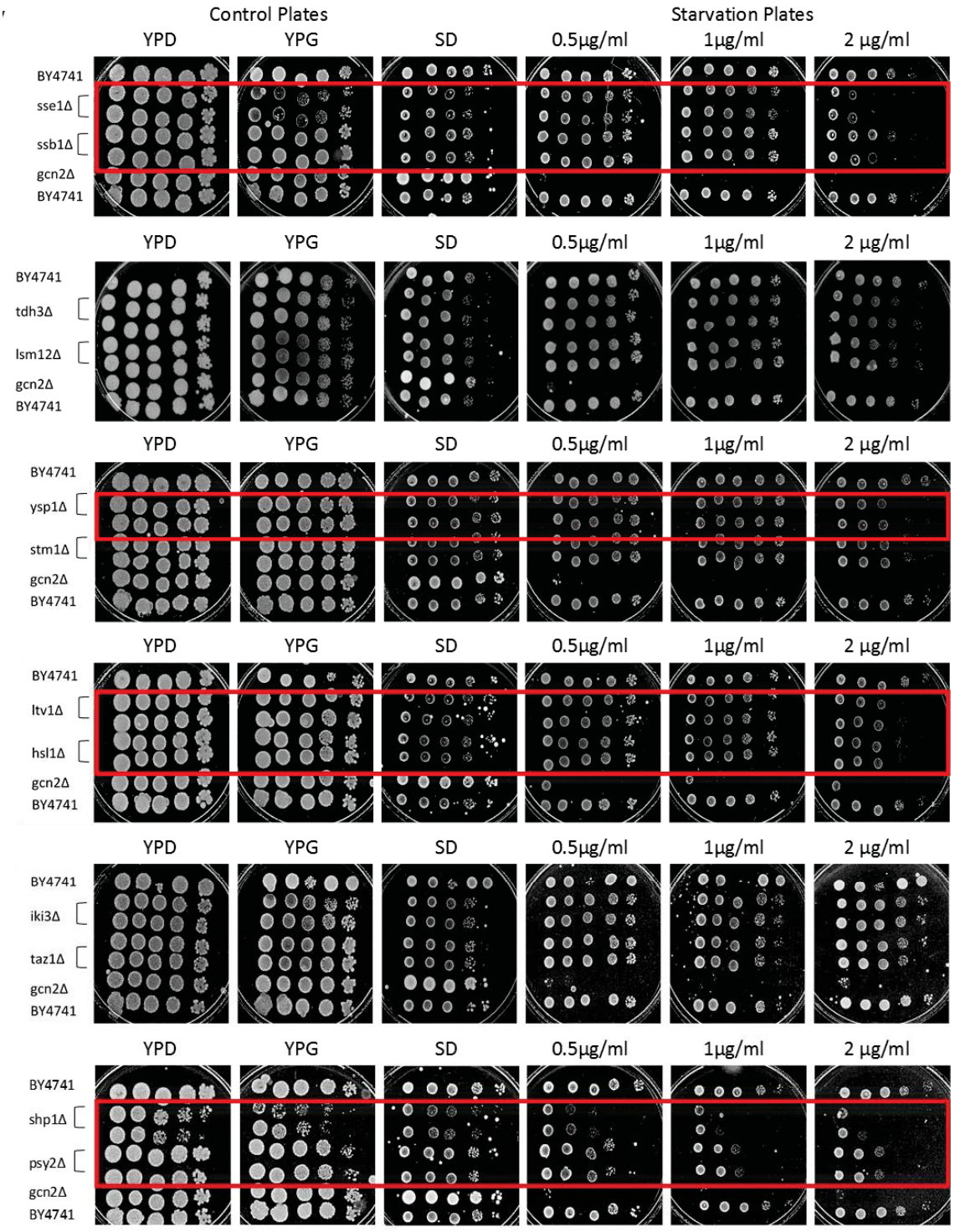
Control Plates
Starvation Plates
YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
sse1Δ
ssb1Δ
gcn2Δ
BY4741
YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
tdh3Δ
lsm12Δ
gcn2Δ
BY4741
YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
ysp1Δ
stm1Δ
gcn2Δ
BY4741
YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
ltv1Δ
hsl1Δ
gcn2Δ
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YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
iki3Δ
taz1Δ
gcn2Δ
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YPG
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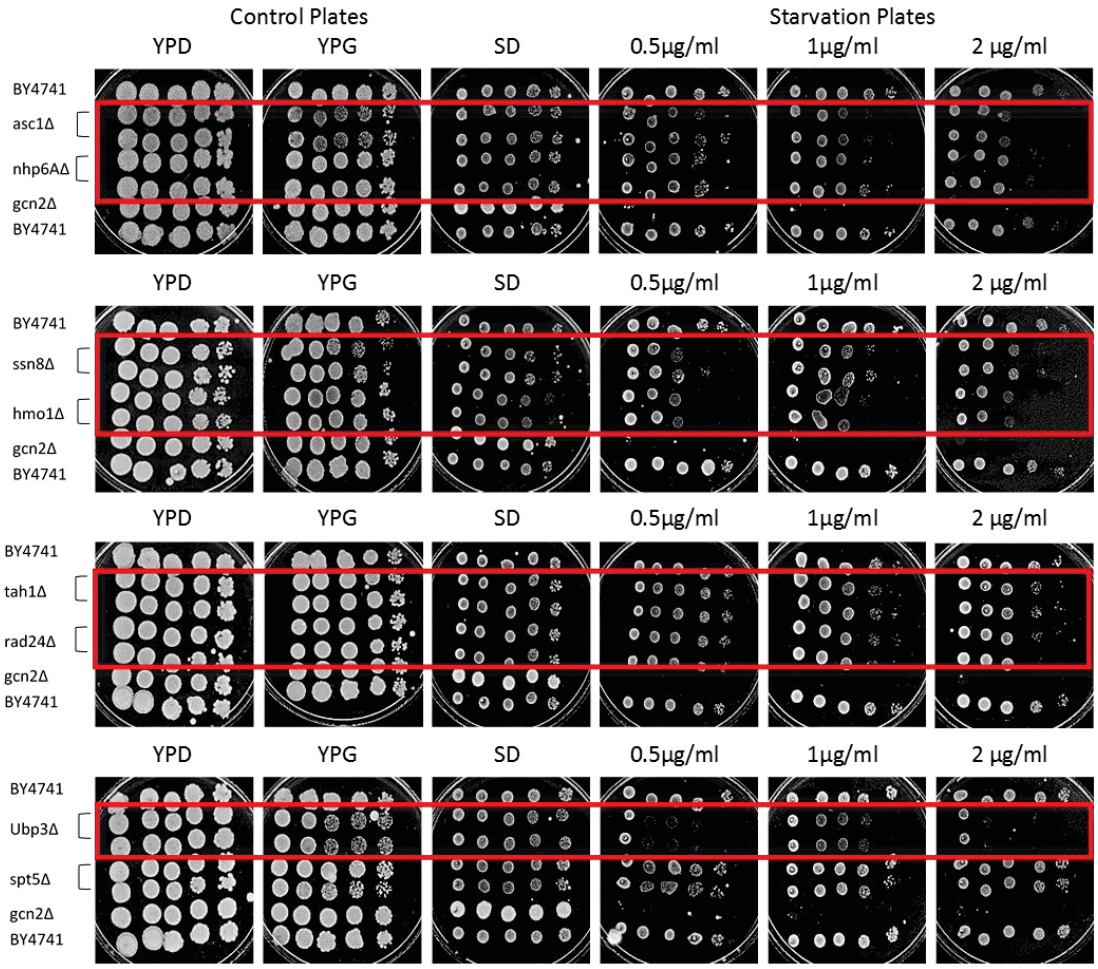
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
shp1Δ
psy2Δ
gcn2Δ
BY4741

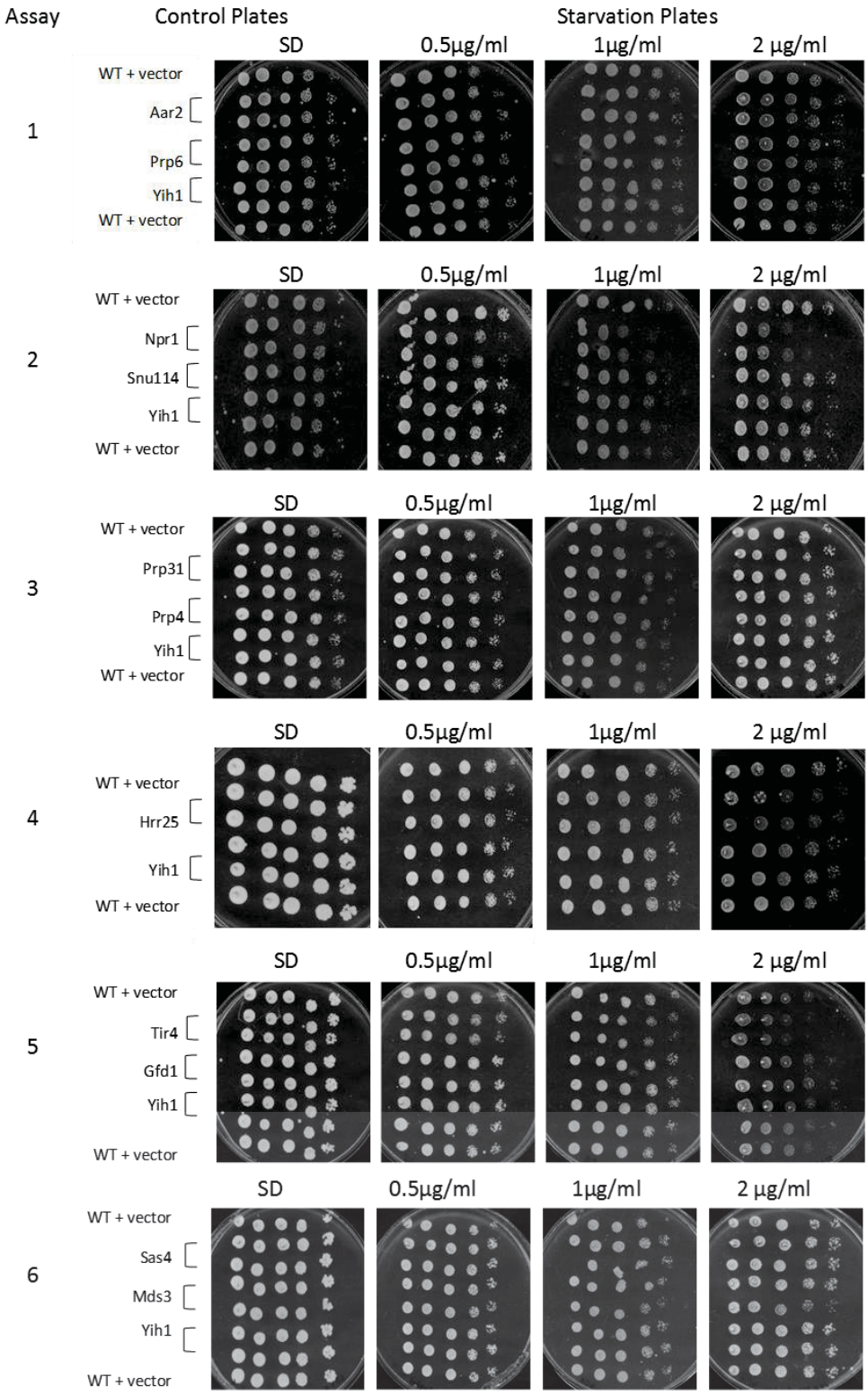

Control Plates
Starvation Plates
YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
asc1Δ
nhp6AΔ
gcn2Δ
BY4741
YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
ssn8Δ
hmo1Δ
gcn2Δ
BY4741
YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
tah1Δ
rad24Δ
gcn2Δ
BY4741
YPD
YPG
SD
0.5µg/ml
1µg/ml
2 µg/ml
BY4741
Ubp3Δ
spt5Δ
gcn2Δ
BY4741

Appendix 2 Growth scores of each strain

Assay	Strain	SD	SM (0.5μg/ml)	SM (1μg/ml)	SM (0.5μg/ml)
1	BY4741	8.5	9	9	9
	kin2Δ	8	8.5	8.5	8.5
	npr1Δ	8	8.5	8.5	8.5
2	BY4741	9	9	9	9
	ask10Δ	9	9	9	9
	fks1Δ	9	9	9	9
3	BY4741	8.5	8.5	8.5	8.5
	boi1Δ	8.5	8.5	8.5	8.5
	haa1Δ	8.5	8.5	8.5	8.5
4	BY4741	9	8	7.5	7.5
	kel1Δ	9	8	7.5	7.5
	kel2Δ	9	8	7.5	7.5
5	BY4741	8.5	8.5	8.5	8.5
	mds3Δ	8	8.5	8.5	8.5
	snf1Δ	8	8.5	8.5	8.5
6	BY4741	9.5	9.5	9.5	9.5
	tor1Δ	9.5	9.5	9.5	9.5
	rgc1Δ	9.5	9.5	9.5	9.5
7	BY4741	9.5	9.5	9.5	9.5
	spo75Δ	9.5	9.5	9.5	9.5
	rtt109Δ	9.5	9.5	9.5	9.5
8	BY4741	9.5	9.5	9.5	9.5
	cmp2Δ	9.5	9.5	9.5	9.5
	pfk2Δ	9.5	9.5	9.5	9.5
9	BY4741	8	8	8	8
	gfd1Δ	7.5	7.5	7.5	7.5
	ubp15Δ	7.5	7.5	7.5	5.5
10	BY4741	9.5	9.5	9.5	9.5
	tir4Δ	9.5	9.5	9.5	9.5
	nba1Δ	9.5	9.5	9.5	9.5
11	BY4741	8	7.5	7.5	7.5
	car1Δ	8	7.5	7.5	7.5
	pph3Δ	8	7.5	7.5	7.5
12	BY4741	8	7.5	7.5	7.5
	hgh1Δ	8	7.5	7.5	7.5
	chs5Δ	7.5	7.5	7.5	5.5
13	BY4741	7.5	7.5	7.5	7.5
	sas4Δ	9	9	9	9
	ncl1Δ	9	9	9	4.5
14	BY4741	7.5	7	7.5	7.5
	rrg9Δ	9	9	9	9
	myo5Δ	9	9	9	9
15	BY4741	6.5	6.5	6.5	6.5

	ymr1Δ	7.5	7.5	7.5	7.5
	bre5Δ	7.5	7.5	7.5	2
16	BY4741	7.5	7.5	7.5	7.5
	rad7Δ	9	9	9	9
	lys20Δ	9	9	9	9
17	BY4741	9	9	9	9
	crp1Δ	9	9	9	9
	rmt2Δ	9	9	9	9
18	BY4741	9	9	9	8.5
	bck2Δ	9	9	9	9
	ede1Δ	9	9	9	9
19	BY4741	9	9	9	9
	spt2Δ	9	9	9	8.5
	pmd1Δ	9	9	9	9
20	BY4741	8.5	8.5	8.5	7.5
	ssa1Δ	7.5	8	7.5	3
	ssa2Δ	7.5	8	8	5
21	BY4741	9	9	9	7.5
	sse1Δ	8.5	9	8	2.5
	ssb1Δ	8	9	8	5
22	BY4741	9	9	9	9
	tdh3Δ	7.5	9	9	7.5
	lsm1Δ	7.5	9	9	7.5
23	BY4741	9	10	10	9
	ysp1Δ	9	9	7.5	6
	stm1Δ	7.5	9	9	7
24	BY4741	8	9	8	8
	ltv1Δ	8.5	9	9	6.5
	hsl1Δ	8.5	9	9	6.5
25	BY4741	10	10	10	10
	iki3Δ	7	9	9	9
	taz1Δ	7	9	9	8.5
26	BY4741	9	9	8	8
	shp1Δ	7	3	2	2
	psy2Δ	8	7	4.5	4.5
27	BY74741	9	9	9	8
	asc1Δ	9	5	5	5
	nhp6aΔ	9	5.5	6	5.5
28	BY4741	9	9	9	9
	ssn8Δ	8.5	5	5	5
	hmo1Δ	7.5	4.5	4.5	4
29	BY4741	9	9	9	9
	tah1Δ	8	6.5	6.0	6.0
	rad24Δ	8.5	7.5	7.5	6.5
30	BY4741	9	10	10	9
	ubp3Δ	9	3	4.5	2
	spt5Δ	9	10	10	9

Appendix 3 Results of SM^s Screen performed on overexpression strains



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