Establishing a screening procedure for finding amino acids important for protein-protein interaction.

A thesis presented in fulfilment of the

requirements for the degree of

Master of Science

in

Genetics

at Massey University, Albany

New Zealand

ADITI ASHOK GHUGE

Aditi Ghuge

2020

Abstract

According to recent findings, the Gcn2 kinase is a global regulator of biological processes in the cell. In *Saccharomyces cerevisiae* (yeast), it has been shown that Gcn2 is activated by its interaction with Gcn1 in the presence of uncharged tRNAs, inducing the starvation response. The binding of Gcn2 to the RWDBD domain of Gcn1 is necessary to activate Gcn2 on medium supplemented with 3AT (3-Amino-1,2,4-Triazole) - which induces starvation for histidine.

In addition, an R2295A mutation in the RWDBD domain prevents the activation of Gcn2 by disrupting the Gcn1-Gcn2 interaction. We predict that the Gcn1-Gcn2 interaction is also mediated by several other amino acids in the Gcn2 binding region of Gcn1. Therefore, to be able to test this hypothesis, a screening method was established in this research that will allow the determination of all possible amino acids in Gcn1 required for Gcn2 binding.

In this thesis we aimed to establish and optimize each module of the screening procedure. One of the modules is competitive growth. This approach will take advantage of the fact that overexpressed RWDBDs in yeast can bind to Gcn2. As a result, the RWDBD disrupts Gcn1-Gcn2 interaction, and prevents Gcn2 activation, thereby preventing yeast from overcoming starvation induced by 3AT. Therefore, yeast will not be able to grow. In contrast to that, yeast strains expressing a mutated RWDBD domain incapable of disrupting the Gcn1-Gcn2 interaction will allow yeast cells to grow on starvation medium. We found that, a competitive growth assay on medium with 0.5 mM 3AT, with a duration of 120 hours, will allow for the enrichment of yeast strains expressing mutated RWDBD domains which have lost the ability to bind to Gcn2.

TRP1 was fused in frame to the C-terminal end of the RWDBD domain which will help to eliminate truncated RWDBD domains from the library of mutated RWDBD domains. The idea was that the presence of a nonsense mutation in the RWDBD domain will lead to the premature termination of translation. As a result, *TPR1* is not translated, and the strain remains auxotrophic for the amino acid tryptophan, whereas RWDBD domains without a nonsense mutation allow the *TRP1* translation, conferring prototrophy for the amino acid tryptophan.

For easy insertion of mutations, the Gcn2 binding region within the RWDBD was flanked with unique new restriction sites, recognized by *AvrII* and *PmeI*. With the help of these new restriction sites, it is now possible to replace the Gcn2 binding region with randomly mutagenized versions. To retrieve the sequences of mutated RWDBD domains easily and efficiently, a cell culture PCR procedure was optimized. In conclusion, in this thesis the major parameters of the screening procedure were optimised.

Acknowledgments

I would like to thank my supervisor Dr. Evelyn Sattlegger for her support, suggestions, ideas, and encouragement throughout the course of my studies. I am thankful for the opportunities she has given me to further my knowledge and abilities with respect to scientific research. I have enjoyed the time spent in the lab and learning how to challenge my own capabilities and overcome any obstacles that have come along the way on my journey so far.

Thanks to all those I share a workspace within our lab and the many new friends I have mad including: Susanne Gottfried, Reuben Anderson, Reagan Dear and Daying Wen. You have all made it an enjoyable experience to work alongside you. Your constant support, ideas, and our scientific discussions have made it easier to understand the complexities of our field of research.

A special thanks to my parents and friends from India who will forever support and encourage me through anything I pursue or come up against no matter how big or how small. A special thanks to Susanne Gottfried, Reuben Anderson, Gabriele Schmidt-Adam, and Gary Ferguson for proofreading my thesis and for their comments which made this thesis possible. A special thanks to Massey university to help me through the COVID-19 pandemic by providing support. A special thanks to Linh Mills for providing me with updated information during this pandemic and making the submission process easy and convenient.

Table of Contents

List of tables

List of figures

List of abbreviations

SD	Synthetic Defined Medium
LB	Luria Bertani media
YPD	Yeast Peptone Dextrose
EDTA	Ethylenediaminetetraacetate
OD	Optical Density
DNA	Deoxyribose Nucleic Acid
TAE	Tris-Acetate-EDTA
3AT	3-Amino-1,2,4-Triazole
SOC	Super Optimal Broth with Catabolic Repression
SOB	Super Optimal Broth
RPM	Rotation Per Minute
SS	Single Stranded
PEG	Ploy ethylene glycol
nm	Nanometre
eIF2α	eukaryotic Initiation Factor 2
Gcn1	General Control Non-Derepressible 1
Gcn2	General Control Non-Derepressible 2
Gcn4	General Control Non-Derepressible 4
BP	Base Pair
MW	Molecular Weight
DD	Double digest
PCR	Polymerase Chain Reaction

RD	Restriction Digestion
ddH_20	Double Distilled water
dNTPS	Deoxy Nucleotide Tri Phosphate
RWD	**R**ING Finger **W**D-repeat containing, **D**EAD helicase
RWDBD	RWD binding domain
eIF2α-P	eukaryotic Initiation Factor 2-Phosphate
tRNA	Transfer RNA
RNA	Ribonucleic Acid
ATF4	Activating Transcription Factor 4
AA	Amino Acid
GST	Glutathione S- Transferase
TRP1	Phosphoribosyl Anthranilate Isomerase TRP1
GAAC	General Amino Acid Control
eEF3	Eukaryotic elongation factor 3
TE	Tris EDTA buffer
$3AT^R$	3AT Resistant
$3AT^S$	3AT Sensitive
ISR	Integrated stress response
RMPCR	Random Mutagenesis PCR
E. coli	*Escherichia coli*
M	Marker or 2log DNA ladder
UC	Uncut
V	Vector
CA	Competition Assay
IMPACT	Imprinted and Ancient *Gene*,

YIH1	Yeast IMPACT Homologous
SLE	Systemic Lupus Erythematosus
SGalWILV	Synthetic defined medium with Galactose

Chapter 1: Introduction

1.1 Amino acids and the eukaryotic cells

Amino acids are the building blocks for proteins. The adequate supply of these amino acids is essential to generate proteins which support the biological function of a cell, its growth, and its development. The cellular level of amino acid needs to be regulated continuously to ensure efficient protein synthesis. Under normal conditions, tRNA (transfer tRNA) binds to their respective amino acid and gets converted to charged tRNA. When the cellular levels of one or more amino acids is low, it leads to what is termed amino acid starvation. This causes an increase in cellular levels of uncharged tRNA.

To sustain an effective protein translation rate, it is mandatory to replenish the cellular levels of amino acids in the cell. Cellular levels of amino acids are replenished by triggering the amino acid starvation response. In eukaryotes, accumulated uncharged tRNA molecules are recognised as a starvation signal, which activates the stress response protein kinase Gcn2 (General control non-derepressable 2) (Padyana, 2005;Hongfang Qiu, 2001 Matthew j. Marton, 1997) . On activation of Gcn2, it phosphorylates its substrate molecule, eIF2 (eukaryotic initiation factor 2). eIF2 is a heteromeric complex consisting of an α, β and γ subunit. The phosphorylation of eIF2 occurs on the α subunit (eIF2α-P). This leads to a reduction in a global protein translation rate, allowing the cell to reduce amino acid consumption during amino acid starvation (Baird & Wek, 2012; Graham d. Pavitt, 1997) At the same time, it increases transcription and translation of mRNA, namely Gcn4 in yeast and ATF4 in mammals (Matthew j. Marton, 1997; Padyana et al., 2005). How the phosphorylation of eIF2 can increase the transcription and translation of Gcn4 and ATF4 while reducing the global translational rate in the starvation condition will be discussed in detail in the following sections.

1.2 Initiation of translation in the eukaryotic cells

The eukaryotic translation initiation factor 2 (eIF2) plays an important role in the initiation of translation in eukaryotes. Initiation of translation begins with eIF2 bound to GTP, forming a ternary complex by binding to $tRNA_i^{Met}$. This ternary complex then binds to the 40S small ribosomal subunit, forming a 43S preinitiation complex (Kimball, 1999). This preinitiation complex binds to the 5' capped structure on mature mRNA and scans mRNA for the start or initiator codon (AUG), which is recognised by the anti-codon of the Met-$tRNA_i^{Met}$ of the ternary complex. Upon reaching the start codon, the 60S large ribosomal subunit joins the pre-initiation complex, allowing the ribosome to commence translation. After initiation of translation, eIF2α-GTP is hydrolysed to eIF2α-GDP. The translational assembly was release once it encounters the stop codon at the end of the mRNA. To initiate the next cycle of translation, eIF2α-GDP is converted to eIF2-GTP through the guanine exchange factor eIF2B (Graham d. Pavitt, 1997). The entire process is illustrated in Figure 1.1

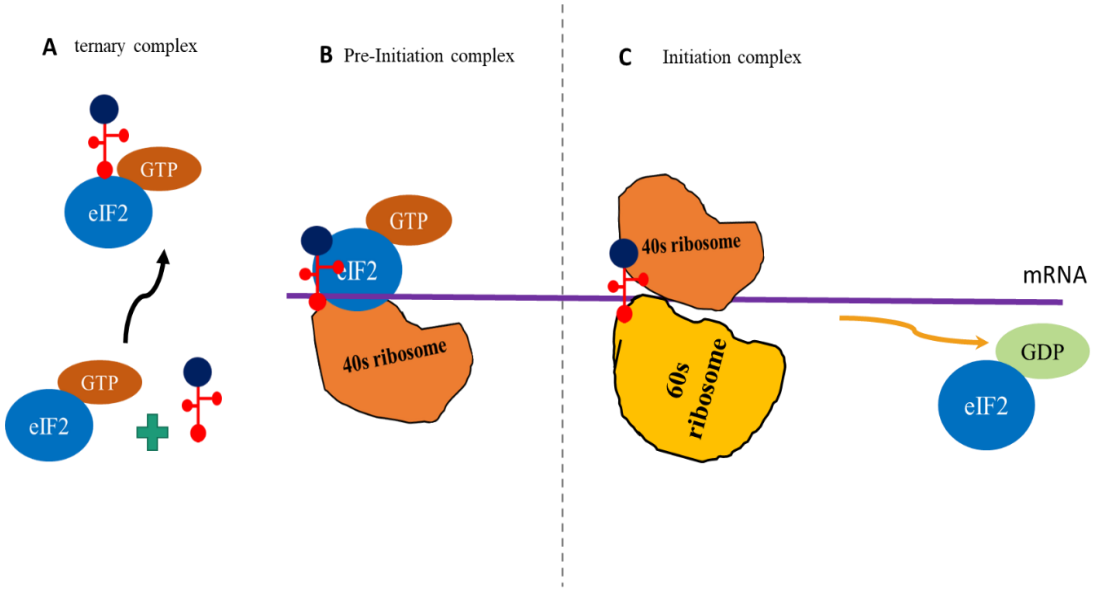


Figure 1. 1 Initiation of protein synthesis.

A. Formation of ternary complex **B**. The newly formed ternary complex attracts the small ribosomal subunit to generate the pre-initiation complex. **C.** The fully functional assembly translates the mRNA, which in turns results in hydrolysis of eIF2-GTP to eIF2-GDP. **E** To initiate the next round of translation, eIF2B converts eIF2-GDP to eIF2-GTP, which can initiate the next round of translation.

1.3 Translational response in the eukaryotic cells during starvation

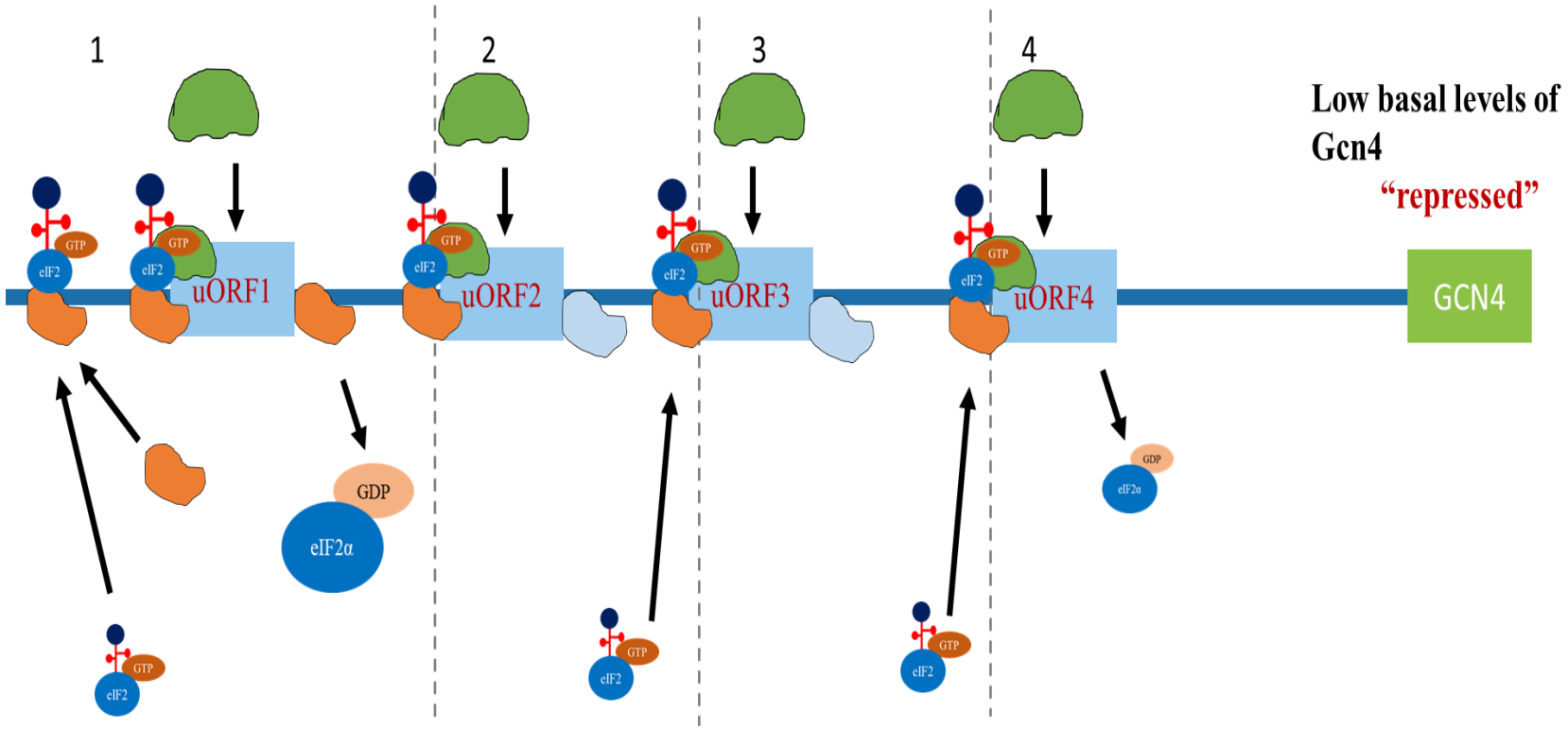
The rate of protein translation is greatly affected by the amino acid response mediated via Gcn2. The phosphorylation of eIF2α by Gcn2 lowers the rate of GTP to GDP exchange on eIF2α (Hinnebusch, 1994). The eIF2α-P acts as a competitive inhibitor for the guanine exchange factor eIF2B and impairs its guanine exchange activity. The phosphorylated eIF2α has an increased affinity towards eIF2B. Therefore, eIF2α-P acts as a competitive inhibitor for non-phosphorylated eIF2α, reducing the cellular concentration of the ternary complex eIF2-$tRNA_i^{Met}$ and the global translation rate (Graham d. Pavitt, 1997; Lageix, Zhang, Rothenburg, & Hinnebusch, 2015).

On the other hand, eIF2α phosphorylation leads to an increased translation of specific mRNA, namely *GCN4* in yeast and the mammalian equivalent ATF4. The *GCN4* mRNA consists of a unique upstream open reading frame (uORF) in the 5' untranslated region of the *GCN4* mRNA. *GCN4* contains four uORF in its mRNA, whereas ATF4 contains two uORF (B. A. Castilho et al., 2014; Graham d. Pavitt, 1997; Matthew j. Marton, 1997).

Under replete / non-starved conditions, the ternary complex, along with the small 40S ribosomal subunits, called the 43S pre-initiation complex, binds to the 5' cap of the mRNA at. The ribosomal subunit then scans for the first start codon, which is the one of the first uORF (uORF1) of *GCN4*. As it reaches the start codon of uORF1 it acquires the large ribosomal subunit (60S) and translation of uORF1 starts. When reaching the stop codon of uORF1, the large subunit dissociates off, and most of the small ribosomal subunits to remain bound to the mRNA. While scanning for the next start codon, the 40S acquires a ternary complex, and translates uORF2. The stop codon of uORF2 is stronger than uORF1 forcing most small ribosomal subunits to dissociate off the mRNA. The same process occurs for uORF3 and uORF4 (Clemens, 1994.; Matthew j. Marton, 1997) (See Figure 1.2A).

As a result, *GCN4* mRNA is barely translated under non-starved conditions meaning that *GCN4* translation is said to be repressed (Clemens, 1994).

A.


1
2
3
4
Low basal levels of Gcn4
"repressed"
GTP
eIF2
uORF1
uORF2
uORF3
uORF4
GCN4
GDP
eIF2α

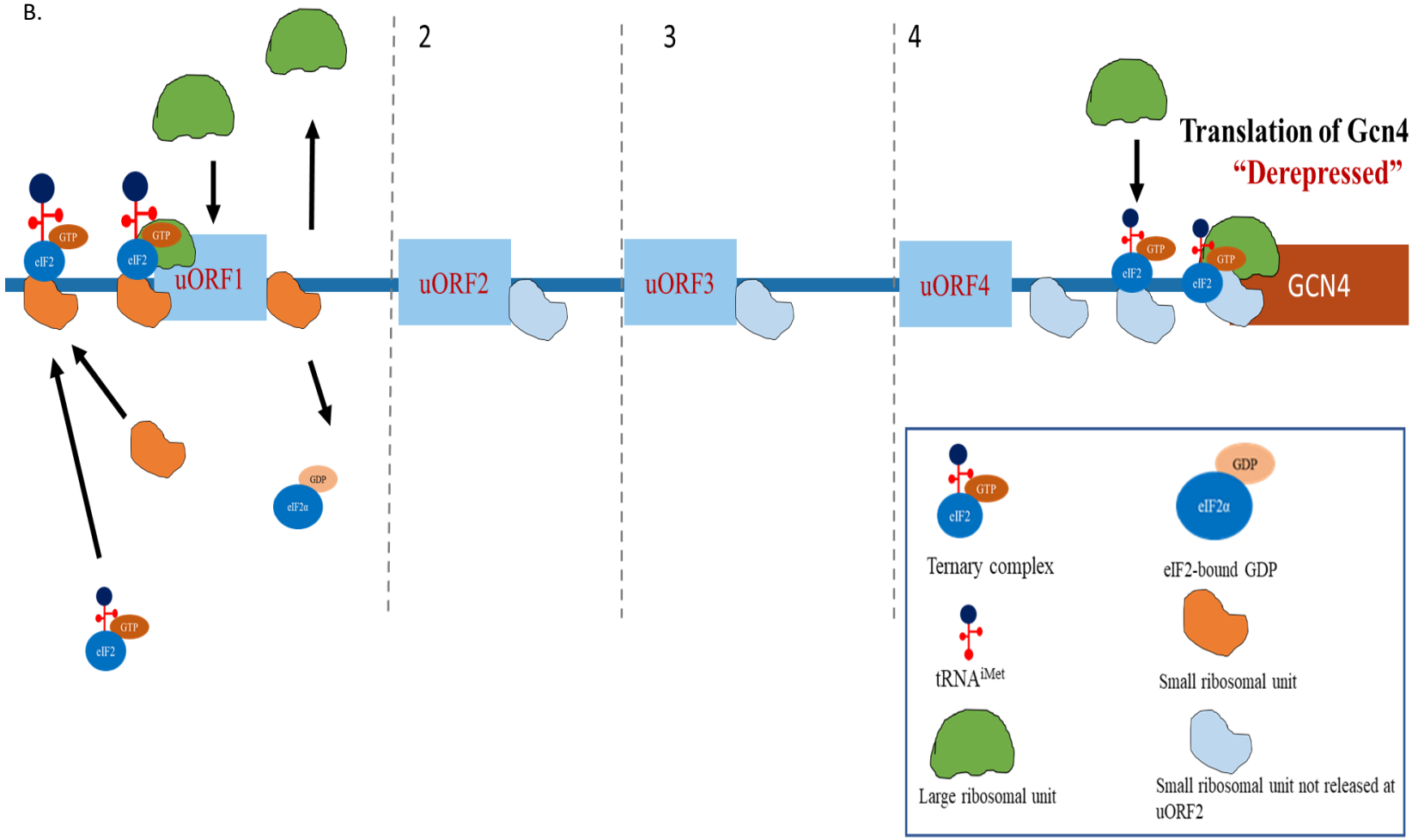

B.
2
3
4
Translation of Gcn4
"Derepressed"
GTP
eIF2
uORF1
uORF2
uORF3
uORF4
GCN4
GDP
eIF2α
Ternary complex
eIF2-bound GDP
tRNA^iMet
Small ribosomal unit
Large ribosomal unit
Small ribosomal unit not released at uORF2

Figure 1. 2 Regulation of *GCN4*

A. ***When GCN4 is repressed during replete condition*** 1. Initiation of protein synthesis occurs as it is illustrated in "Figure 1.1 initiation of protein synthesis". Only the difference here is the presences of stop codon at uORF1, which is a weak stop codon. on reaching a stop codon, it results in dissociation of the elongation assembly, allowing the small 40S ribosomal subunits to stay attached to the mRNA. **2**. The attached small 40S ribosomal subunits acquires the ternary complex and forms the initiation complex with the large ribosomal unit and translates uORF2. The stop codon of uORF2 is stronger than uORF1, allowing the translational assembly to dissociate entirely from the mRNA. Consequently, Gcn4 is not translated, meaning its translation is repressed. **3 and 4.** Any bound small ribosomal subunit will be eliminated by translating uORF3 and uORF4 in a replete condition. Moreover, it prevents the translation of Gcn4, keeping it in a repressed condition. **B *when Gcn4 is derepressed during the non-replete condition*** the only difference here is the stop codon of uORF1 is a weak stop codon, which results in partial dissociation of the translational assembly, allowing the small ribosomal unit to stay attached to the mRNA. **2,3 and 4.** Due to low cellular concentrations of ternary complex, the bound small ribosomal unit fails to acquire the ternary complex; consequently, it is dissociated from mRNA at the strong stop codon of uORF2. Consequently, uORF3 and uORF4 remain untranslated. 4. Moreover, the distance between the uORF4 and mRNA of Gcn4 is bigger than the other uORFs which allows far more time for the bound small ribosomal subunit to acquire a ternary complex and the large ribosomal subunit before it reaches the start codon of Gcn4. This ultimately leads to translation of GCN4 during a starvation condition. The translation of Gcn4 under these conditions is then called "derepressed". The image is adaptation of (Carlos R.Vazquez de Aldana, 1995)

Under amino acid starvation conditions, the cellular levels of the ternary complex are reduced, consequently, reducing the translational rate at uORF (Figure 1.3 B). This allows the bound ternary complex along with the small ribosomal subunit to scan through uORF2 uORF3 and uORF4. Moreover, the distance between uORF4 and the *GCN4* mRNA is larger than the other uORFs. This allows time for the pre-initiation complex to get converted into an initiation complex before it reaches the start codon of *GCN4* which ultimately leads to translation of *GCN4* during a starvation condition. The translation of *GCN4* under these conditions is consequently named "derepressed", due to the reversal of the repressed state occurring under replete conditions (figure 1.3B){Carlos R.Vazquez de Aldana, 1995, Hinnebusch, 2005}.

In conclusion, the cellular concentration of the ternary complex regulates the translational rate of *GCN4*. During amino acid starvation, Gcn2 phosphorylates eIF2α, which is able to reduce to the cellular levels of the ternary complex, leading to increased *GCN4* translation and helps yeast cells to overcome the amino acid starvation by regulating the transcription and translation of *GCN4* mRNA to activate the Gcn2 starvation response (Hinnebusch, 2005).

1.4 Gcn2: Domain and their function

The protein kinase Gcn2 (See Figure 1.3) is a large protein found to be highly conserved in eukaryotes (Mathew j. Marton, 1993). Gcn2 is an eIF2α kinase which plays an essential role in amino acid starvation (see above "1.3 translational response in a eukaryotic cell during starvation"). Gcn2 is activated during amino acid starvation via the accumulation of uncharged tRNA molecules within the cell. Gcn2 has several domains: A protein kinase domain, the pseudokinase domain (which shares structural homology with protein kinase domain but lacks critical amino acids making it an enzymatically inactive domain, (see Figure 1.3), the **R**ING figure **W**D-repeat containing protein **D**EAD-like helicase like (RWD) domain which interacts with Gcn1(an effector molecule of Gcn2), a His-RS (**His**tidyl- t**R**NA **S**ynthetase) domain, and the C-terminal domain (CTD) (See Figure 1.3) (Dong et al., 2000; Qiu, Hu, Dong, & Hinnebusch, 2002)

The His-RS domain and the CTD of Gcn2 govern the binding of uncharged tRNAs to Gcn2 (see Figure 1.4). After the binding of uncharged tRNA, Gcn2 undergoes an allosteric re-arrangement to carry-out an auto-phosphorylation of amino acids present in the activation loop of proteinkinase domain (Hongfang Qiu, 2001). Upon activation of Gcn2, it is then able to phosphorylate its substrate molecule eIF2α. During non-starvation conditions, Gcn2 is inhibited by auto inhibitory molecules such as eEF1A (Visweswaraiah et al., 2011). A mutation in the His-RS domain of Gcn2 has been shown to reduce its affinity towards tRNAs, which leads to reduced phosphorylation of eIF2α, suggesting that the His-RS domain is essential for detecting the starvation signal (Manuel ramirez, 1992; Padyana et al., 2005). (G.Hinnebusch1, 2000; sheree a. wek, 1995)

The N-terminal 127 amino acid of Gcn2 consists of the RWD domain which is the binding site for the effector molecule, Gcn1(Hinnebusch, 2000; Minerva Garcia-Barrio,2000). The interaction of uncharged tRNAs with Gcn2 is suggested to be highly dependent on the complex formation between Gcn2 and Gcn1. Amino acids 1-125 of the RWD domain are sufficient for binding to Gcn1 (G.Hinnebusch1, 2000; Kubota, Ota, Sakaki, & Ito, 2001; Kubota, Sakaki, & Ito, 2000; Ma1thew j. Marton, 1993; Rakesh, Krishnan, Sattlegger, & Srinivasan, 2017) The interaction of Gcn2 with Gcn1 is essential for the *in vivo* activation of Gcn2 (G.Hinnebusch1, 2000).

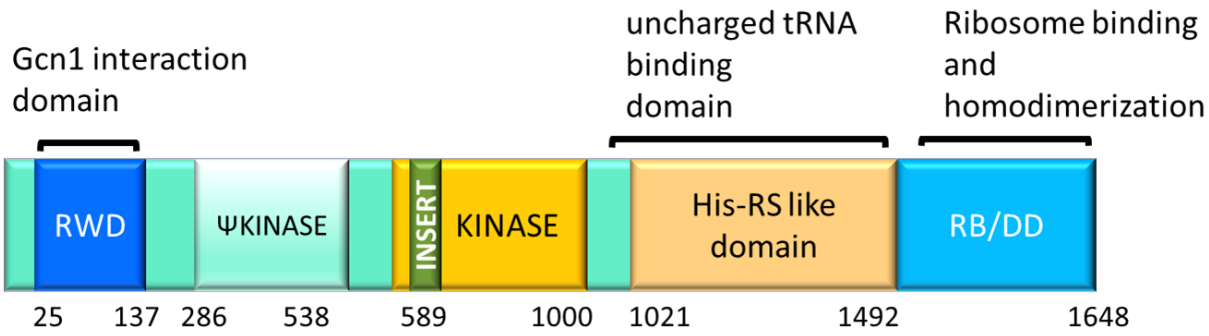


Figure 1. 3 Diagrammatical representation of Gcn2.

The RWD domain interacts with Gcn1. The pseudokinase domains inhibit the phosphorylation of the protein kinase domain under non-starvation conditions. The His-RS domain is involved in transferring the uncharged tRNA molecule to Gcn2 in starvation conditions. The CTD (RR/DD) domain is involved in ribosome binding and homodimerization.

1.5 Gcn1 structure and their function

Gcn1 (Figure 1.4) is 2672 amino acids long protein.(Kubota et al., 2000; Rakesh et al., 2017). Gcn1 is present in all eukaryotic organisms, and its function is suggested to be conserved between all eukaryotes. Gcn1 is an effector molecule of Gcn2 which is essential for the activation of Gcn2 during the amino acid starvation response. Gcn1 binds to Gcn2 through a domain called the RWD binding domain (RWDBD), consisting of amino acids 2052 to 2428 of Gcn1 (see Figure 1.4) (Sattlegger & Hinnebusch, 2000) Deletion of Gcn1 (*gcn1*Δ) in yeast leads to absolutely no phosphorylation of eIF2α *in vivo*, and completely inhibits the ability of yeast to grow on starvation media (M. Marton, Crouch, & Hinnebusch, 1993). However, Gcn1 is not required for the catalytic activity of Gcn2 in yeast, as the cell extracts a *gcn1*Δ strain shows eIF2alpha phosphorylation (M. Marton et al., 1993). A constitutively activated mutant of Gcn2 ($Gcn2^c$) cannot activate the amino acid starvation response in a *gcn1*Δ strain, suggesting that the function of Gcn1 is to transfer the uncharged tRNAs to Gcn2 as opposed to Gcn1 to begin the requirement for the Gcn2 enzymatic activity *per se* (Kubota et al., 2001; M. Marton et al., 1993; Qiu, Hu, Dong, & Hinnebusch, 2002).

The amino acids located to N-terminus of Gcn1, have shown to be involved in ribosomal binding (see Figure 1.4). The interaction between Gcn1 and ribosome is essential for activation of Gcn2 (Inglis et al., 2019; Matthew j. Marton, 1997). Furthermore, M1A and M7A mutations in Gcn1 shows reduced ability of Gcn1 to interact with the ribosome, significantly affecting the activation of Gcn2 under starvation conditions. The middle portion of the N-terminal domain of Gcn1 consists of an eEF3 binding-like domain (see Figure 1.4). The eEF3-like domain has HEAT repeats (the acronym arising from the presence of these repeats in the proteins **H**untingtin, e**E**F3, protein phosphatase **A**, and **T**or) which is suggested to allow the binding of other proteins to Gcn1(Matthew j. Marton, 1997).

Furthermore, the mutation in the RWDBD (C-terminal arm domain of Gcn1, see Figure1.4) at Arginine 2259 to alanine (R2259A) weakens its interaction with Gcn2, suggesting that the R2259 is crucial for the Gcn1-Gcn2 interaction. (G.Hinnebusch1, 2000; Kubota et al., 2001)

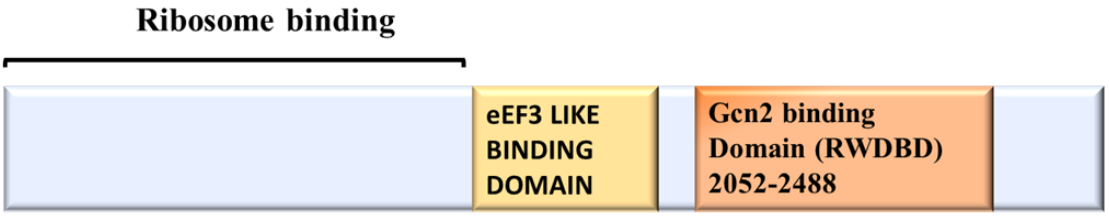


Figure 1. 4. Diagrammatical representation of study for Gcn1

The RWD binding domain interacts with Gcn2. The ribosome binding region interacts with ribosomal protein. The eEF3-like domain allows the interaction between Gcn2 and the ribosomal protein.

1.6 Gcn1 - Gcn2 Interaction

As mentioned in the above section, for Gcn2 to be activated, it must interact with Gcn1. The studies conducted and published by Sattlegger et al. (2000) showed that the N-terminus of Gcn2 and amino acids 2052-2428 of Gcn1 are mainly involved in mediating the Gcn1-Gcn2 interaction. Yeast strains containing Gcn1 that lack area D, now known as RWDBD domain were unable to grow on medium supplemented with 3AT. This mutated Gcn1 was also unable to co-precipitate Gcn2. This suggests that the RWDBD is crucial for Gcn1-Gcn2 interaction (see Figure 1.5). Furthermore, when the R2259 in Gcn1, located within the RWDBD, was substituted by Ala, these yeast strains are not able to grow under starvation conditions. This suggested that the R2259 of Gcn1 was critical for Gcn2 activation. In the same yeast strain, when Gcn2 was overexpressed under starvation conditions, this strain could again overcome starvation and grow. This suggested that more amino acids than R2259 are involved in mediating the Gcn1-Gcn2 interaction.(Hinnebusch, 2000; Kubota et al., 2001)

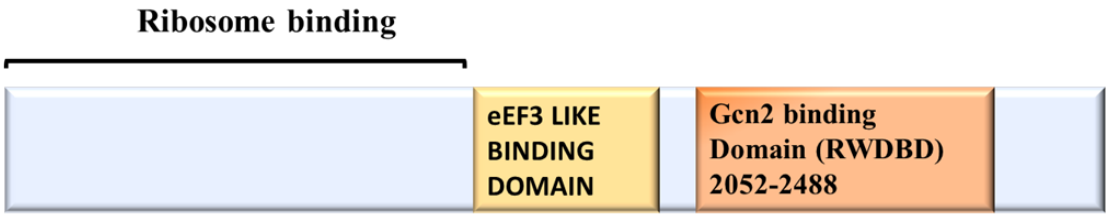


Figure 1. 5 Diagrammatical representation of study for Gcn1.

When the RWDBD domain was overexpressed in yeast cells, it could not overcome the starvation and could not grow on 3AT plate. This indicated that the overexpressed RWDBD disrupted the interaction between Gcn1 and Gcn2. Whereas when the RWDBD domain with R2259A mutation was overexpressed in yeast cells, the strain could overcome the starvation and grow on a 3AT plate. This suggested that Gcn1 interacts with Gcn2 through RWDBD and the interaction between RWDBD and Gcn2 is essential for its function and activation. The yeast two hybrid study by Kubota et al (2000) showed that the yeast cells expressing the truncated fragment of amino acids (2064-2382) of Gcn1 could not grow on medium lacking

histidine. This suggested that these amino acids were required for Gcn2 binding. However, this area is different to that found by Sattlegger et al 2000. One would assume that the area in common between those two regions is the one harbouring the Gcn2 binding site *per se*. As one would expect, this does contain R2259 (G.Hinnebusch1, 2000).

Kubota et al. (2001) conducted a screen to identify amino acids in Gcn1 required for Gcn2 binding. They found that F2291L (phenylalanine to leucine), S2304P (serine to proline) and L2353P (leucine to proline) mutations resulted in defective Gcn1-Gcn2 interaction. The yeast strain expressing F2291L and L2353P mutations could not overcome the starvation on a 3AT plate. Furthermore, when yeast strains expressing F2291L and L2353P were grown under starvation conditions and analysed for eIF2α phosphorylation through immunoprecipitation, they showed a significant reduction in eIF2α phosphorylation. This suggested that these are mutations which are essential in mediating the Gcn1-Gcn2 interaction (Kubota et al., 2001; Rakesh et al., 2017). However, the recently published study on the structure of the RWDBD published by Rakesh et al 2017 suggests that F2291L and L2353P mutations cause the destabilisation of the helix-helix structure of Gcn1, causing a change in the 3D structure of Gcn1 (see Figure 1.6). This suggested that they are essential for maintaining the 3D structure of Gcn1 but are not essential for Gcn2 binding *per se*. Another mutation, S2304P (serine to proline), causes defective interaction with Gcn2 and preventing the activation of Gcn2 in starvation condition. The Gcn1 protein (see Figure 1.6). This suggested that the mutations found in the study by Rakesh et al 2017 do not serve as a direct contact point for Gcn2 binding and are mainly involved in maintaining the 3D structure of Gcn1(Rakesh et al., 2017).

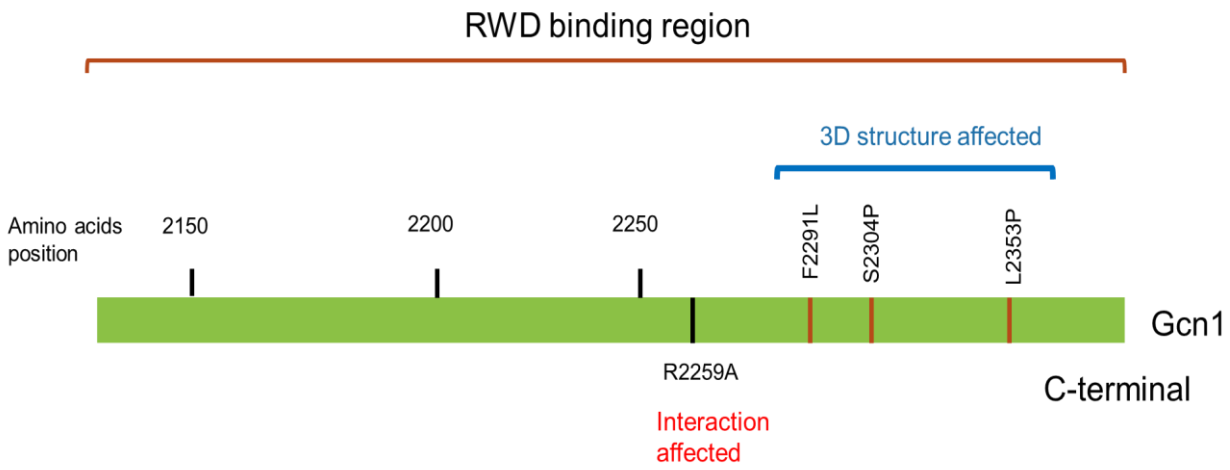


Figure 1. 6 Overview of Gcn1 mutations affecting the Gcn1-Gcn2 interaction.

R2259A mutation directly affects the Gcn1-Gcn2 interaction, whereas F2291L, S2304P and L2353P affect the 3D structure of Gcn1, not the Gcn1-Gcn2 interaction. This image is adapted from Kubota et al. 2001.

In contrast, the R2259A mutation of RWDBD domain, which was found to be directly involved in the Gcn1-Gcn2 interaction, was not be found in the mutational screen conducted by Kubota et al. 2001 (see Figure 1.6). This suggests that the screening by Kubota was not comprehensive and that they likely may have missed other amino acids required for Gcn2 binding.

If R2259 were the only contact point between Gcn1 and Gcn2, the yeast strain expressing R2259A-RWDBD would not be able to grow on starvation medium when Gcn2 was overexpressed. This very strongly suggests that there are more amino acids than R2259 that are involved in mediating the Gcn1-Gcn2 interaction, and that their identity is still unknown. Therefore, in this thesis, we aim to establish a procedure to comprehensively screen for amino acids involved in mediating the Gcn1-Gcn2 interaction.

1.7 Proposed signalling mechanism of Gcn2 activation.

The signalling pathway leading to the activation of Gcn2 has been extensively studied in yeast, leading to a proposed mechanism for its activation during amino acid starvation. However, many vital steps in this pathway remain to be fully elucidated. Research so far led to a working model of how Gcn1 transfers uncharged tRNA onto Gcn2 (see Figure 1.7) (Beatriz A Castilho et al., 2014; Lee, Swanson, & Sattlegger, 2015; M. J. Marton, Vazquez de Aldana, Qiu, Chakraburtty, & Hinnebusch, 1997; Ramirez, Wek, & Hinnebusch, 1991; Evelyn Sattlegger & Hinnebusch, 2000).

The working model proposes that to activate Gcn2 fully, Gcn2 must form a trimeric complex with Gcn1 and the ribosome (see Figure 1.7). Moreover, Gcn2 and Gcn1 need to interact for Gcn2 to be activated. They interact with each other via unique interacting domains. In Gcn2, the domain is called RWD, and in Gcn1 it is the RWD-Binding-Doman (RWDBD) domain (Evelyn Sattlegger & Hinnebusch, 2000).

It is suggested that the accumulated uncharged tRNAs are first transferred to ribosomal A-site (acceptance site) and then transferred to Gcn2's His-RS domain (Carlos r. Vazquez de aldana, 1994,; Lageix et al., 2015). The model suggests that Gcn1 plays a crucial role in the transfer of uncharged tRNAs to Gcn2. Once the uncharged tRNA is transferred onto the His-RS domain of Gcn2, Gcn2 undergoes an allosteric re-arrangement, phosphorylates its substrate molecule eIF2α (see Figure 1.7) and activates the starvation response.(James C. Jiang, 2000)

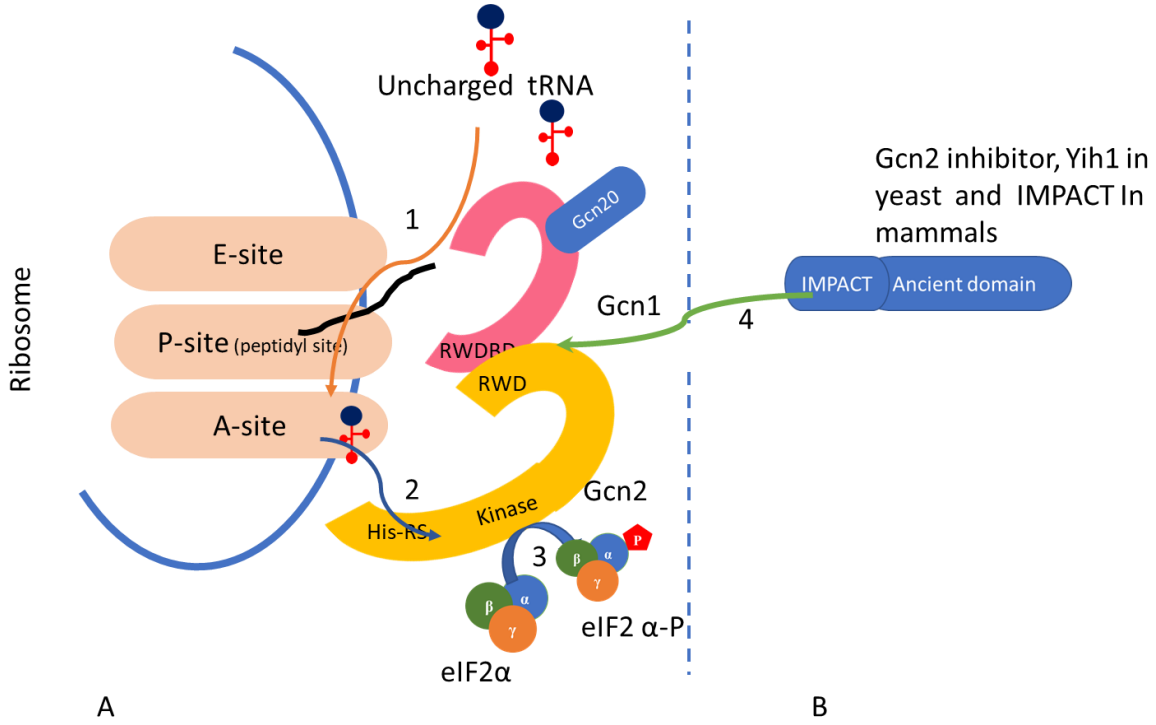


Figure 1. 7 Proposed signaling mechanism of Gcn2 activation.

A. During amino acid starvation the uncharged tRNA molecule is recognised as the starvation signal. This uncharged tRNA is transferred to the His RS-like domain of Gcn2 through Gcn1-Gcn20 complex at the ribosomal A-site (denote as 1 and 2). After the uncharged tRNA was recognised by the His RS-like domain, the kinase domain phosphorylates its substrate molecule eIF2 at its alpha subunit, and thereby activates the starvation response (denoted by 3).

B. During non-starved conditions, the activity of Gcn2 is inhibited by an YIH1 inhibitor (in yeast) and IMPACT (in mammals) which abolishes the interaction between Gcn1-Gcn2. This image is adapted from (B. A. Castilho et al., 2014; Roffe, Hajj, Azevedo, Alves, & Castilho, 2013), denoted as 4.

1.8 Research hypothesis

The amino acid starvation response requires the presence of Gcn1 to activate Gcn2. For Gcn2 to be activated, it must interact directly with Gcn1. The interaction between Gcn1 and Gcn2 is mediated by amino acids present in the RWD domain of Gcn2 and the RWDBD domain of Gcn1. As mentioned in section 1.6, the Gcn1-Gcn2 interaction, we know that the amino acid R2259A mutation in the RWDBD domain of Gcn1 is crucial for the Gcn1-Gcn2 interaction. (G.Hinnebusch1, 2000)

Because the mutation of R2259 did not totally abolish, but instead weakened, the Gcn1-Gcn2 interaction, I hypothesise that more than just R2259 in the RWDBD domain is involved in mediating the Gcn1-Gcn2 interaction. To be able to test this hypothesis here, a screening method will be established that will allow the identification of amino acids of the RWDBD domain which mediates binding to Gcn2.

1.9 Aim of this study

This research aims to develop a screening procedure that allows the identification of amino acids in the RWDBD domain of Gcn1 that are required for mediating its interaction with the RWD domain of Gcn2.

1.10 Generalised idea for fast identification of amino acid present in RWDBD crucial for Gcn2 binding.

The general idea of the screening process is outlined here and illustrated in Figure 1.8

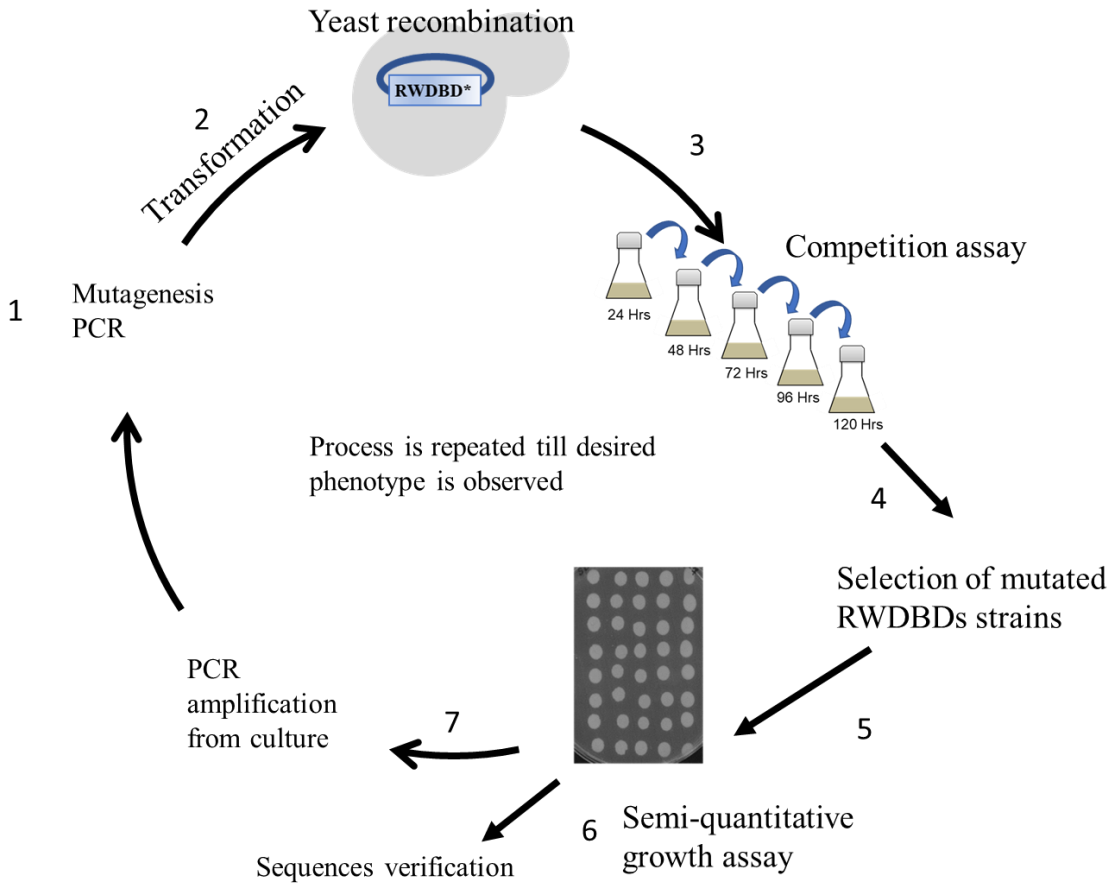


Figure 1. 8 Overview of screening procedure to enrich mutated RWDBDs

In step **1**. the mutations were introduced in the RWDBD domain through random mutagenesis PCR. **2**. These mutated RWDBD domains were then transferred to a yeast cell along with a cut open vector through transformation. Recombination. **3**. The pool of transformants are then subjected to competitive growth in medium containing 3AT. **4.** Selection of only those strains who has lost the ability to bind to Gcn2 binding region. **5**. Selected strains were analysed for growth on 3AT plate through semi-quantitative growth assay. **6.** The strains showing strong 3AT resistance will be selected and the sequence will be verified. **7.** When yeast cells failed to show strong 3AT resistance, to insert more mutations, the RWDBDs domain is amplified from liquid culture via PCR. Amplicon used as a template to introduce more mutations through random mutagenesis PCR, and the entire process is repeated until the all the strains are expressed as mutated RWDBD.

Mutagenesis PCR can be utilised for an easy insertion of mutations into RWDBD using a random mutagenesis approach (see Figure 1.8 step 1). This mutated RWDBD domain along with a cut open vector will be transformed into a yeast cell to generate plasmids through homologous recombination that express the mutated RWDBD (see Figure 1.8 step 2). The population of yeast transformants, each containing an RWDBD with a different mutation(s), will be subjected to competitive growth under starvation conditions (see Figure 1.8 step 3). When mutated RWDBDs are overexpressed under starvation conditions, only those will be selected which have lost the ability to bind to Gcn2. As a result, yeast strains expressing mutated RWDBDs will be enriched. (see Figure 1.8 steps 4 and 5).

An aliquot of the enriched strains will be plated to select several individual yeast colonies, and their ability to grow under starvation conditions will be tested (see Figure 1.8 step 5). This is to test whether the enrichment was successful. The next step is to sequence the RWDBD expressed in those strains to identify their mutations (see Figure 1.8 step 6)

Most likely the Gcn2 binding may not be fully abolished after only one round of random mutagenesis. In this case another round of mutagenesis will be conducted. For this, the region of the RWDBD will be PCR amplified directly from the enriched culture (see Figure 1.8 step 7), followed by another cycle of random mutagenesis, and the entire procedure of transformation and competitive growth is repeated until at least 90% of the cells show re-gained ability to activate Gcn2.

To conduct this screening procedure, it is necessary to optimise several steps in this process. This is the aim of this thesis. To achieve our goal, we have optimised five components of this screening procedure. They are listed below.

1.11 Objectives

1. **Optimisation of a system that selects for RWDBD that have lost the ability to bind to Gcn2.**

We will take advantage of the fact that the overexpressed RWDBD impairs Gcn2 activation and thus reduces growth under starvation conditions, but a mutated RWDBD unable to bind Gcn2 does not impair the Gcn2 function and allows the yeast strain to grow during starvation conditions.

2. **Generating a plasmid that allows the easy insertion of random mutations into the Gcn2 binding region of the RWDBD**

For this we will introduce restriction sites into the RWDBD domain that flank the Gcn2 binding region.

3. **Optimisation of random mutagenesis.**

Random mutagenesis will be done via error prone PCR. We will establish conditions that will lead to 1-2 random mutations in the Gcn2 binding region.

4. **Developing a system which removes truncated RWDBD domains from the library of mutated domains**

For this, an enzyme essential for tryptophan biosynthesis will be fused in-frame to the C-terminal end of the RWDBD. A nonsense mutation, leading to truncated RWDBD and consequently to the lack of translation of the enzyme, would not confer tryptophan resistance to the cell and therefore not allow the cell to grow on medium lacking tryptophan.

5. **Retrieval of sequences of mutated RWDBD that have lost their Gcn2 binding ability.**

To retrieve the sequences of all enriched mutated RWDBDs via PCR, with as little bias as possible from the yeast cell culture, in this thesis the PCR amplification process was optimized. Since the template is liquid cell culture, this PCR reaction is called liquid cell culture PCR. Due to time constraints this objective could not be performed.

1.12 Scoring of Gcn2 activity

In this section, we aim to explain how the Gcn2 activity can be scored when using yeast cells overexpressing RWDBDs. To score for Gcn2 activity during the starvation condition, we utilise the fact that Gcn2 is only functional when the Gcn1-Gcn2 interaction is established and not disrupted.

In this thesis, as mentioned, mutated RWDBDs will be overexpressed in the yeast cells to determine whether they have lost the ability to bind to Gcn2 binding regions or not. To determine whether the introduced mutation is crucial for the Gcn1- Gcn2 interaction, we need an easy and fast way to score for Gcn2 activity.

The Gcn2 activity in cells can be easily scored on starvation medium, where cells unable to activate Gcn2 are hardly able to grow. In contrast to that, when Gcn2 is active in a cell, it allows the yeast to grow on the starvation medium (see Figure 1.9).

In this thesis, we aim to overexpress RWDBDs in yeast cells. When mutated RWDBDs are overexpressed that have lost the ability to bind to Gcn2, this will allow the yeast to grow in starvation conditions by restoring the Gcn1-Gcn2 interaction (see Figure 1.10 B). As a result, yeast cells can grow on the starvation medium. On the other hand, when wildtype RWDBD is overexpressed in yeast, it can bind to Gcn2 and can disrupt the Gcn1-Gcn2 interaction. Consequently, Gcn2 cannot be activated. Thus, cells are unable to grow (See Figure 1.10 A).

To conclude, the ability of yeast cells to growth under starvation conditions can be used as an indicator to score whether Gcn2 can be activated or not.

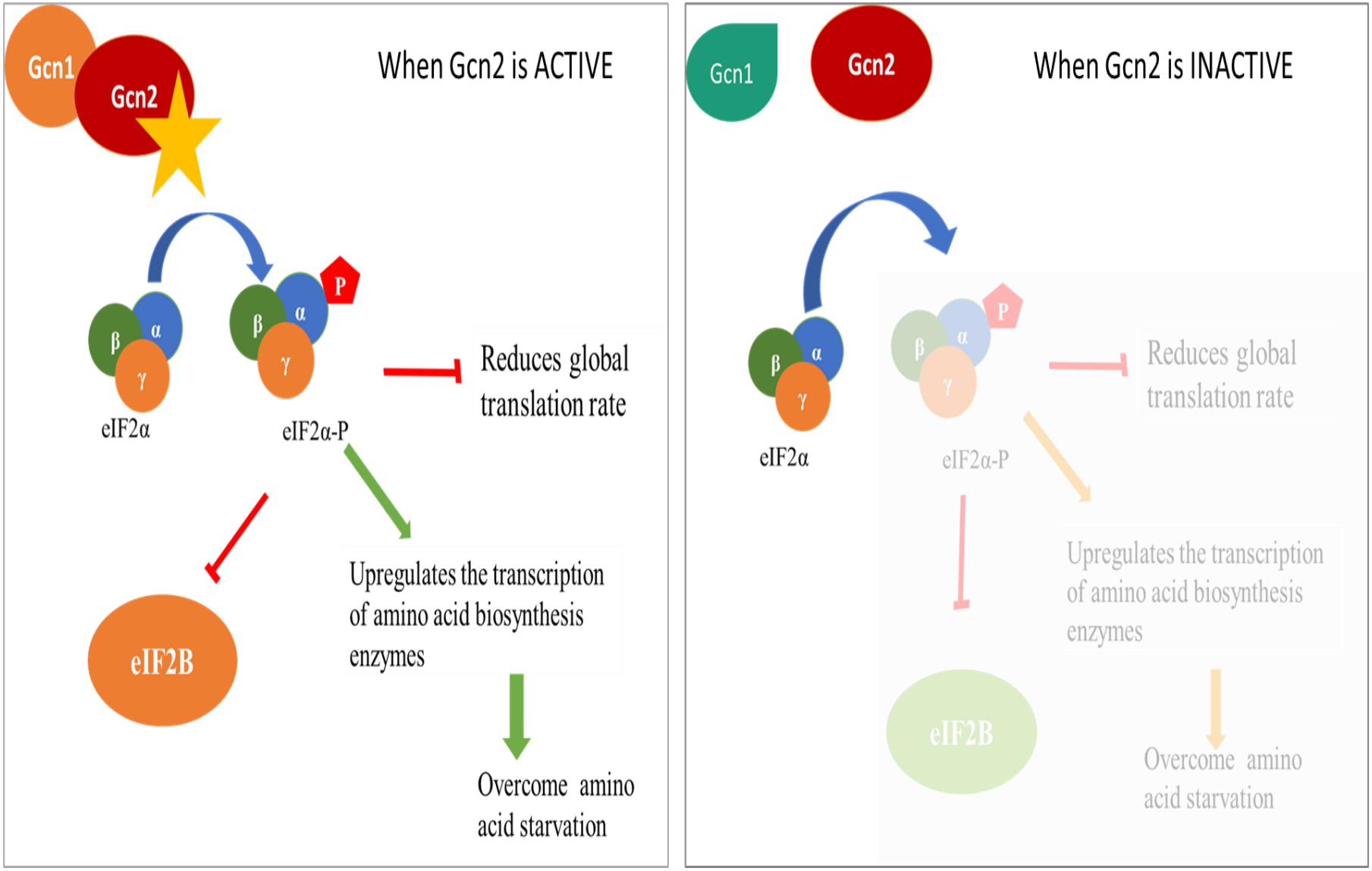

When Gcn2 is ACTIVE
Gcn1
Gcn2
P
eIF2α
eIF2α-P
Reduces global translation rate
eIF2B
Upregulates the transcription of amino acid biosynthesis enzymes
Overcome amino acid starvation
When Gcn2 is INACTIVE
Gcn1
Gcn2
eIF2α
eIF2α-P
Reduces global translation rate
Upregulates the transcription of amino acid biosynthesis enzymes
eIF2B
Overcome amino acid starvation

Figure 1. 9 Gcn2 function and amino acid starvation response in a yeast cell.

When Gcn2 is functional: During amino acid starvation, Gcn2 gains its functions via interacting with Gcn1. After phosphorylation of eIF2α, Gcn2 reduces the global translational rate, impairs the activity of eIF2B, and increases the synthesis of the amino acid biosynthesis enzyme, which allows the yeast cell to cope with the starvation response and to grow on the starvation medium. When Gcn2 is not functional.

When Gcn2 is non-functional it fails to gain its function due to the disrupted Gcn1-Gcn2 interaction. Since the interaction between Gcn1 and Gcn2 is disrupted, Gcn1 fails to transfer the uncharged tRNA molecule to the His-Rs domain, allowing Gcn2 to remain in an inactive form. As a result, the yeast cell fails to cope with the starvation response and is unable to grow on the starvation medium.

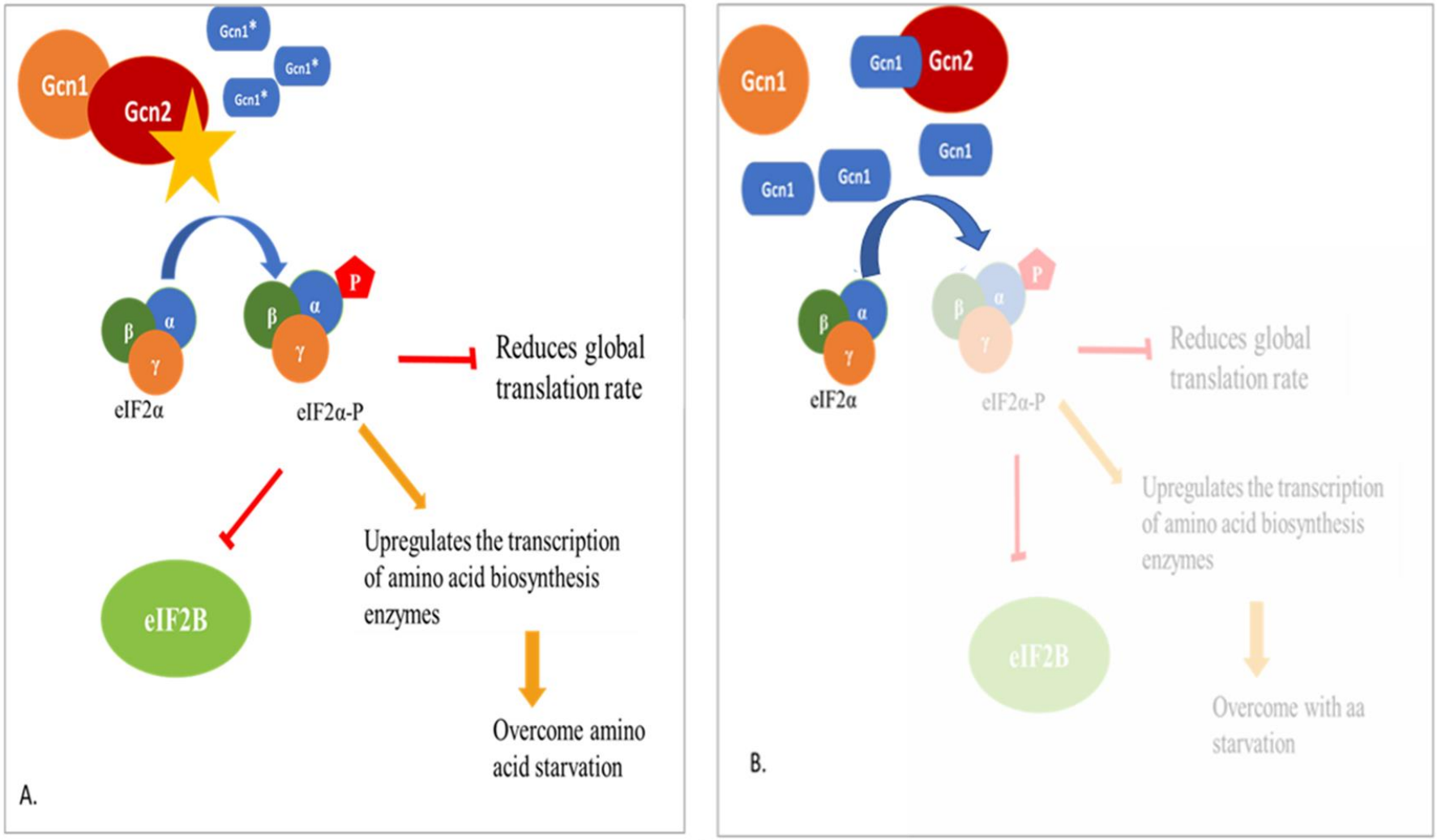

Gcn1
Gcn2
Gcn1*
Gcn1*
Gcn1*
P
eIF2α
eIF2α-P
Reduces global translation rate
eIF2B
Upregulates the transcription of amino acid biosynthesis enzymes
Overcome amino acid starvation
A.
Gcn1
Gcn2
Gcn1
Gcn1
Gcn1
P
eIF2α
eIF2α-P
Reduces global translation rate
eIF2B
Upregulates the transcription of amino acid biosynthesis enzymes
Overcome with aa starvation
B.

Figure 1. 10 Gcn2 function and amino acid starvation response when RWDBD domain is overexpressed in a yeast cell.

A The overexpressed RWDBD acts as a competitive inhibitor for Gcn1 and prevents its interaction with Gcn2 by blocking the RWD domain on Gcn2. AS the interaction between Gcn1-Gcn2 is disrupted, Gcn2 loses its function, failing to activate an amino acid starvation response. Therefore, yeast cells are unable to grow on a starvation medium.

B. When the mutated RWDBD domain is overexpressed in the yeast cell during the starvation condition, Gcn2 gains its function and activates an amino acid starvation response. The gain of function is observed due to transfer of uncharged tRNA to the His-Rs domain of Gcn2 by Gcn1, indicating that Gcn1 can interact with Gcn2 through the RWDBD domain, and could overcome the starvation and grow.

1.13 Relevance of research

Besides its function of keeping amino acid homeostasis in the cell, Gcn2 is also implicated in several diseases and disorders. Recent investigations have shown that the Gcn2 kinase acts as a memory regulator for short-term and long-term memory response. Furthermore, in Alzheimer's disease, Gcn2 helps to prevent memory loss (Devi & Ohno, 2013). Moreover, Gcn2 acts as a cell checkpoint regulator of the cell during the stress condition (Grallert & Boye, 2007).

Furthermore, Gcn2 is one of the crucial key players to mediate immunological response. Gcn2 helps to induce immunological response and helps to prevent the spread of infection during viral and bacterial attacks (Berlanga et al., 2006; Liu et al., 2014). In cancer biology, Gcn2 helps cancer cells to overcome starvation caused by increases in nutrient uptake (Koromilas, 2015). Gcn2 also helps to suppress the inflammatory response by increasing production of the anti-inflammatory cytokine IL-10 (Interleukin 10), TGF- β. Moreover, Gcn2 also triggers T cells to undergo apoptosis and lowers the formation of inflammasomes.(Carroll, Korolchuk, & Sarkar, 2015; Liu et al., 2014; Ravishankar et al., 2015)

To conclude, Gcn2 plays important roles in regulating various biological functions; thus, it is important to study the activation pathway of Gcn2. As mentioned in sections 1.12 and 1.6, to fully activate the Gcn2 function, it needs to interact with Gcn1, a co-factor molecule essential for Gcn2 function and activation (E. Sattlegger et al., 2011). Studies suggests that the Gcn1-Gcn2 interaction is also required for all the other biological functions Gcn2 is implicated in. To date, very little research has been conducted on the Gcn1-Gcn2 interaction. Therefore, studying this interaction and identifying the key amino acids involved in mediating this interaction will help us to determine a fragment which could be used as a pharmacologically active agent for treating Gcn2-associated diseases/disorders, such as cancer and various immunological disorders (Carroll et al., 2015; Ravishankar et al., 2015).

Chapter 2. Materials and Methods

2.1 Yeast strains used

Table 2. 1: Yeast strains used in this study

Strain	Genotype	Source
H1151	*Ura3-52, trp1-63, leu2-3, leu2-11, gal2*$^{+}$	Foieni et al. 1991
H2256	(*Gcn1Δ*)	C. R. Vazquez de Aldana and A. G. Hinnebusch

2.1.2 Escherichia coli strain used in the study

Table 2. 2: Escherichia strain used in this study

Strain	Genotype	Source
DH5α	-	Hanahan, 1983

2.2 Plasmids used in this study.

Table 2. 3: Plasmid used in this study

Plasmids	Markers	Source
pES128-9-1(GST)	GST alone	E. Sattlegger and A.G Hinnebusch (2000)
pES124-B2(GST-RWDBD-myc)	URA3, *leu2-d*, 2µ	E. Sattlegger and A.G Hinnebusch (2000)

pES124-B3(GST-RWDBD-TRP1-myc)	URA3, *leu2-d*, 2µ, *TRP1*	In this study
pES167-2E (GST-RWDBD^{R2259A}-myc)	URA3, *leu2-d*, 2µ	E. Sattlegger and A.G Hinnebusch (2000)
pES701/703	*Trp1, URA3*	In this study
pES167-2E-1(GST-RWDBD^{R2259A}-TRP1-myc)	URA3, *leu2-d*, 2µ, *TRP1*	In this study
pAG01(GST-RWDBD^{R2259A}-TRP1-myc)	URA3, *leu2Δ,* 2µ, *TRP1*	In this study
pAG02(GST-RWDBD-TRP1-myc)	URA3, *leu2-d*, 2µ, *TRP1* AvrII and PmeI restriction site added	In this study

2.3 Media compositions

All the growth media and solutions, unless mentioned otherwise, were prepared using double de-ionised water (Milli Q). All the components and media solutions were sterilised via heating at 121 °C and 15 psi pressure for 20 minutes or passing through a 0.2-micron cellulose acetate filter paper under vacuum conditions (Sartorius Stedim Biotech). All the reagents used in the preparation of solutions and media are listed in appendix table 5.8

- **Liquid media (Broths)**

  The liquid media were prepared by dissolving all components in MilliQ water and sterilised through autoclaving. The media supplements were added under aseptic conditions. The media were stored at room temperature. The medium with added drug/antibiotic was stored at 4°C.

- **Solid medium (Agar medium)**

The solid media were prepared the same as the liquid media, except that agar was added. Furthermore, 25 mL of the medium was poured into Petri dishes and allowed to solidify overnight and stored at 4 °C.

- **Glucose and Galactose stock solutions**

  80 g of glucose (FORMEDIUM™) and galactose (FORMEDIUM™), respectively, were dissolved in up to 200 mL of double de-ionised water to prepare a 40% stock solution of sugar.

- **YPD (Yeast Peptone Dextrose (1 litre)**

Table 2. 4: Composition of YPD medium

Yeast extract (FORMEDIUM™)	10 g
Peptone (FORMEDIUM™)	20 g
Agar (FORMEDIUM™)*	20 g
Distilled water	Up-to 1000 mL
40% Glucose (FORMEDIUM™)	50 mL

- *Added only for solid media

- **Luria Brentani media (LB) (1 litre)**

Table 2. 5: Composition of LB medium

Bacto Tryptone (FORMEDIUM™)	10 g
Yeast extract (FORMEDIUM™)	05 g
Sodium chloride (Ajax)	10 g
Agar (FORMEDIUM™)*	20 g
Distilled water	Up-to 1000 mL

- *Added only for solid media

- **Synthetic defined Medium (SD) (1 litre)**

Table 2. 6 Composition of SD medium

Yeast Nitrogen Base (YNB) (FORMEDIUM™)	1.9 g
Ammonium sulphate (FORMEDIUM™)	05 g
40% Glucose / Galactose** (FORMEDIUM™)	50 mL
ILV stock solution (FORMEDIUM™)	20 mL
Tryptophan stock solution (FORMEDIUM™)	10 mL
Agar (FORMEDIUM™)*	20 g
Distilled water	Up-to 1000 mL

- *Added only for solid medium, galactose** is used for protein overexpression

- **Super optimal broth (SOB) (1 litre)**

Table 2. 7: Composition of SOB medium

Yeast extract (FORMEDIUM™)	5 g
Tryptone (FORMEDIUM™)	20 g
Sodium chloride	0.5 g
250 mM KCl	10 mL
2M $Mgcl_2$	5 mL
pH	7
Distilled water	Up-to 1000 mL

- **Super optimal catabolic repression (SOC) (1 litre)**

Table 2. 8: Composition of SOC medium

Yeast extract (FORMEDIUM™)	5 g

Tryptone (FORMEDIUM^{TM})	20 g
Sodium chloride	0.5 g
20 mM glucose	2 mL
250 mM KCl	10 mL
2M $Mgcl_2$	5 mL
pH	7
Distilled water	Up-to 1000 mL

2.4 Stock solutions

- Amino acid stock solution

The amino acid tryptophan was filter sterilised and stored in a lightproof container at 4 ºC. Isoleucine, Leucine and Valine (ILV) stock solution was prepared together and was sterilised through autoclaving.

Table 2. 9 Concentration of amino acids used in this study

Amino acid	Solvent	Stock concentration	Final concentration (g/100 mL)	mL/L	mL/plate
Leucine (FORMEDIUM^{TM})	De-ionized water	50 mM	1.31		
Isoleucine (FORMEDIUM^{TM})	De-ionized water	100 mM	0.656	20 mL	0.2 mL
Valine (FORMEDIUM^{TM})	De-ionized water	50 mM	0.586		
Tryptophan* (FORMEDIUM^{TM})	De-ionized water	40 mM	0.8	10 mL	0.1 mL

2.5 Drugs used

- 3-Amino-1,2,4-triazole stock solution (2 M):

3-Amino -1, 2, 4-triazole stock solution was prepared freshly and sterilised by filtration.

Table 2. 10: Drugs used in this study

Drug	Solvent	Final concentration (g/100 mL)
3-Amino-1,2,4-triazole* (FORMEDIUM^{TM})	MiliQwater	16.816 g/100 mL

- Ampicillin stock solution

Ampicillin was filter sterilised and stored at -20 ^{o}C for further use.

Table 2. 11: Antibiotic used in this study.

Antibiotic	Solvent	Final concentration (µl/100 mL)
Ampicillin (FORMEDIUM^{TM})	MiliQwater	50

2.5 Growth conditions

- For yeast cells

The yeast saturated culture was generated by growing the appropriate yeast strains in sterile YPD or SD medium (liquid medium) along with supplements depending upon the requirement of the strains at 30 °C at 150 revolutions per minutes (rpm) in aerobic conditions.

Yeast colonies were preserved by streaking them onto a selective medium (solid medium) in an aseptic condition, growing them at 30 °C for 48 hours and stored at 4 °C.

- For *Escherichia coli*

The saturated *Escherichia coli* (*E. coli*) culture was prepared by inoculating a single colony of a strain in sterile liquid LB medium supplemented with ampicillin. The culture was grown at 37 °C shaking at 180 rpm in aerobic conditions.

E. coli colonies were preserved by streaking onto LB (solid medium) under aseptic conditions and growing them at 37 °C for 16 hours. Plates were stored at 4 °C.

- Long term storage conditions for a yeast culture

30% glycerol was prepared by mixing 30 mL of glycerol to 70 mL of water. 2-3 yeast colonies were picked from a plate and inoculated in the 700 µL sterile 30% glycerol stock solution and stored at -80 °C.

- Long term storage condition for bacterial culture (transformed)

A transformed overnight bacterial culture was added to 100% glycerol (700 µL of glycerol with 250-300 µL of *E. coli* culture) and stored at -80 °C.

2.6 Yeast transformation

- Preparation of competent cells.

A single colony of yeast wild type strain H1151 was inoculated in rich medium YPD grown overnight at 30 °C. 1 mL of the overnight culture was inoculated in 50 ml of YPD medium and allowed to grow to OD_{600} of 0.8 at 30° C shaking. After reaching an OD_{600} of 0.8, cells were harvested via centrifugation at 4000 rpm for 5 min at 4 °C. The pellet was resuspended in 8 ml solution I and incubated for 30 min shaking at 30 °C (Budding yeast: a laboratory manual)

- Transformation

The competent yeast cells were pelleted by centrifugation at 4000 rpm and resuspended in 500 µL of solution I. To an Eppendorf's tube 5 µL plasmid DNA (80-100 ng), 5 µL ssHerring sperm (80-100 ng) (allows the easy insertion of foreign DNA) followed by 100 µL competent cells were mixed by gentle pipetting and incubated for 30 min at 30 °C. After incubation 700 µL of solution II was added and incubated for 45 min at 30 °C shaking. Then cells were

subjected to heat-shock treatment at 42 °C and incubated for 10 min and then on ice for 5 minutes. The cultures were then centrifuged and resuspended in SD medium and plated on selective medium and incubated for 2-4 days.

- Solution 1

Solution	Volume (final concentration of 1x)
10 X TE pH 7.4	1 mL
1M lithium acetate in TE	1 mL
Water	8 mL

- 10 X TE (10 x is concentration)

1.211 Grams of Tris and 372.24 g of EDTA were dissolved in 100 mL of water and pH was adjusted to 7.4 using concentrated HCl.

- 1 M Lithium acetate

10.2 g of lithium acetate was dissolved in 10 mL of 10 X TE then the volume was made up to 100 ml. The solution was sterilised by filter sterilisation.

- Solution 2

Solution	Volume
44% PEG (Sigma) in 1X TE	9 mL
1M Lithium acetate (sigma) in 1 X TE	1 mL

- 44% PEG in 1X TE

44 g of polyethylene glycol was dissolved in 10 mL of 10 X TE then the volume was made up to 100 mL and sterilised by autoclaving at 121 °C for 20 minutes

2.7 Escherichia coli transformation

A. Preparation of competent cells.

- By chemical method

The strain DH5α was inoculated in LB (luria bertani) rich medium and allowed to grow overnight. 1 mL of an overnight culture was reinoculated in 50 mL of LB medium and allowed to grow up to OD of 1.5 at 37° C shaking. After reaching an OD of 1.5, cells were harvested via centrifugation at 3000 rpm for 10 min at 4 °C. The pellet was resuspended in 20 ml of $CaCl_2$ (50 Mm ice-cold solution) and incubated for 30 minutes on ice. After incubation, a pellet was generated and resuspended in 4 mL of $CaCl_2$ solution. An aliquot was made and rapidly frozen with dry ice and stored at -80°C.

- Preparation of electrocompetent cells

1 mL of an overnight culture of DH5α E. *coli* was re-inoculated into 100 mL of LB medium and incubated at 37 °C in shaking condition until an OD_{600} of 0.6 was reached. Once the culture was at OD_{600} of 0.6, the flask was immediately submerged into ice-water slurry and immediately cooled down via vigorous shaking for 5 minutes. After quick initial chilling of cells, the flask was incubated on ice water slurry and shaken occasionally. After incubation, the cells were transferred to prechilled 50 mL falcon tubes and centrifuged at 4200 rpm for 10 minutes. The excess medium was discarded. The pellet was resuspended in 25 mL of prechilled 10% glycerol solution and incubated for 10 min on ice. After incubation, the cells were centrifuged at 4200 rpm and resuspended in 10% glycerol solution. After centrifugation, the cell pellets were joined and resuspended into 500 µL of 10% ice-cold glycerol solution. An aliquot of 70 µL was made and rapidly frozen using dry ice and stored at -80 °C.

B. transformation

- By chemical method

An aliquot of competent cells was thawed on ice. After thawing 2-3 µL of the plasmid DNA was mixed, and cells were heated at 42 °C for 30 seconds and immediately placed on ice for 5 minutes. After incubating on ice, the cells were incubated at 37 °C for an hour in 1 mL of regeneration medium (SOC). After incubation, the cells were plated onto a selective medium and incubated for 17 hours at 37 °C (Boeringer Mannheim: Protocol “molecular biology” 1987).

- By electroporation

An aliquot of the competent cells was removed from -80 °C and thawed on the ice for 5 min. After thawing 1 µL of plasmid, ligation product was added and the cells electroporated at 1850 Volts. Immediately after electroporation, 500 µL of warm SOC medium was added to allow the cells to recover at 37 °C in shaking conditions. 100 µL of the culture was plated on the selective medium and incubated for 18 hours at 37 °C. Micropulser™, Bio-Rad™ (Dower, W. J., Miller, J. F., and Ragsdale, C. W. (1988))

C. Reagents used

- 1 M stock solution of $CaCl_2$

14.70 grams of solid $CaCl_2$ (Sigma) was dissolved in 100 mL of deionised water and autoclaved at 121 °C for 20 minutes.

- 10% glycerol solution

100 mL (V/V) of glycerol in 900 mL of deionised water was autoclaved at 121 °C for 20 minutes.

2. 8 Growth curves

The growth curve was performed for the wild-type and Gcn1 deletion strain by inoculating them in SGalWILV medium supplement with all amino acid. A different 3 AT concentrations (0.025, 0.5, 0.5, 1, 5 30, 60, 90 and 120 mM) was added to the medium, and O.D. was measured every 2 hours at 600 nm. A graph of O.D. versus time was plotted in the semi-logarithmic pattern. The same procedure was followed for the following strains GST-RWDBD-TRP1-myc and GST-RWDBD*-TRP1-myc and wild-type and Gcn1 deletion strain. A different 3 AT concentration was used to study the behaviour pattern of strains in liquid media and O.D. was measured every two hours at 600 nm. A graph of absorbance versus time was plotted in semi-logarithmic fashion (budding yeast: a laboratory manual).

2.9 Competitive growth assay

To perform the competitive growth, assay the H1151 wild type strain was transformed with a plasmid containing construct 1 and construct 2 individually. See the following constructs.

- Construct 1 is GST-RWDBD-TRP1-myc $^{LEU2\Delta}$, having leu2 deletion hence leucine auxotrophic (unable to grow without amino acid leucine)
- Construct 2 is GST-RWDBD ^{R2259A}-TRP1-myc, leucine prototrophy (able to grow without amino acid leucine)

Both the constructs are under control of a galactose inducible promoter (see figure 3.1).

To simplify the understanding of competition assay, construct 1 was referred to as a wildtype strain and construct 2 as a mutated strain. Strains containing construct 1(wildtype) and construct 2 (mutated) were grown to saturation in SGluWILV (supplemented with glucose) medium. To initiate competition assay, 9 (90 µL) parts of construct 1 was mixed with 1 (10 µL) part of construct 2 to reach a starting OD_{600} of 0.5. This mixed culture was inoculated to two flasks containing selective medium (SGalWILV), one containing 0.5 mM of the starvation inducing drug 3 AT and the other control flask without 3 AT. Flask were incubated shaking at 30 °C for up to 24 hours. After 24 hours of incubation, the optical density of each flask was determined. If the optical density exceeded more than 0.2, then 500 µL of culture was inoculated to fresh medium. If the OD_{600} was under 0.2, 1 mL of culture was added to a fresh flask. After every 24 hours of incubation, the ratio of construct 1 (wildtype) to construct 2 (mutated) was determined via plating a 10-fold serially diluted culture onto a selective solid medium (SGluWILV and SGluW) and allowed to grow for 48 hours at 30 °C. After 48 hours of incubation, the number of colonies was counted, and the ratio of construct 2 to construct 1 was calculated as follows. Counted colonies were multiplied by the dilution factor to determine the number of cells in the initial culture. To normalise the obtained data, the cell number was expressed relative to the cell count of the plate where all the cells were able to grow. The numbers were expressed as per cent.

2.10 Semi-quantitative growth assay (SQGA)

Previously transformed colonies were inoculated with a selective medium containing 2 % glucose and grown to saturation at 30 °C. A ten-fold serial dilution (1 to 1/10000) was made for each culture, and 5 µL of each dilution was transferred onto starvation and non-starvation solid media [SGluWILV, SGalWILV, starvation medium with different concentration of 3 AT (10 mM, 30 mM and 60 mM and 90 mM, 120 mM)] and incubated at 30° C. Growth was monitored over a period of 20 days. The colony growth on the plates was scanned using

enhanced Microtek scanning systems (Microtek RealSkan systems, Budding yeast: a laboratory manual)

2.11 Polymerase chain reaction (PCR)

The polymerase chain reaction is a molecular technique used to amplify out DNA fragments from whole genomic or plasmid DNA. The fragment of interest is amplified using a small DNA oligomer commonly known as a primer (20-25 nucleotide sequences, forward and reverse). The primers were used in conjunction with heat-stable polymerase in the thermocycler. The polymerase chain reaction is mainly distributed into three steps discussed as follows.

1. Denaturation: The template was heated to 94-95 °C to convert double-stranded DNA into single-stranded DNA for the annealing of the primers in the next step.
2. Annealing/hybridisation: The template was cooled down to 55-65 °C for the annealing of the primer to single-stranded DNA.
3. Extension/ Amplification: The amplification of a fragment of interest was carried out via a heat-stable DNA polymerase. The fragment of interest was amplified.

The three steps were repeated cyclically 30-35 times to amplify the PCR product. The standard PCR reaction volume was 20 µL and was prepared as follows. The addition of heat stable polymerase (*kapa or Pfx*) depends on the type of PCR reaction.

Table 2. 12 A standard PCR reaction mix

Component	Volume
PCR-grade water	Up to 20 µL
10 X *pfx* buffer	2 µL
50 mM $MgSo_4$	0.4 µL
10 mM dNTP Mix	0.4 µL
10 µM Forward primer	0.6 µL
10 µM Reverse primer	0.6 µL
5 U/µL Taq DNA polymerase	0.2 µL
Template DNA	0.4 µL

Table 2. 13: Cycling protocol for PCR reaction

Step	Temperature in °C	Duration	Cycles
Initial denaturation	94 °C	5 minutes	1
Denaturation	94 °C	15 seconds	
Annealing	55 °C	30 seconds	35
Extension	68 °C	90 seconds	
Final Extension	68 °C	90 seconds	1
Hold	4 °C	∞	

2.11a Cell culture Polymerase chain reaction

Cell culture PCR is a version of PCR that allows the amplify the gene of interest from whole yeast cells, eliminating the requirement of pure DNA samples. The colony PCR was performed in the same manner as described earlier. A small amount of fresh yeast cells was added to the PCR reaction instead of plasmid DNA

Table 2. 14 Master mix for colony PCR

Component	Volume
PCR-grade water	Up to 20 µL
10 X *kapa* buffer	2 µL
50 mM $MgSo_4$ *	1.2 µL
10 mM dNTP Mix	0.4 µL
10 µM Forward primer	0.8 µL
10 µM Reverse primer	0.8 µL
5 U/µl *kapa* Taq DNA polymerase	0.08 µL
Cell culture	1 µL

The PCR reaction mix, along with the cell culture was spun down prior to the PCR reaction analysis. The cell culture PCR reaction was carried out as follows.

*only added if necessary

Table 2. 15 : A cycling protocol for cell culture PCR

Step	Temperature in °C	Duration	Cycles
Initial denaturation	94 ^oC	5 minutes	1
Denaturation	94 ^oC	30 seconds	
Annealing	55 ^oC	30 seconds	35
Extension	72 ^oC	60 seconds	
Final Extension	72 ^oC	60 seconds	1

Hold	4 °C	∞	

2.11b Fusion Polymerase chain reaction

Fusion PCR is a technique used to produce a fusion DNA fragment from fragments of DNA through overlap sequencing without the addition of restriction sites or DNA ligase enzyme. In this PCR method, a fragment is added as a template DNA along with an appropriate primer sequence to achieve the full-length PCR product. The thermocycler is designed as mentioned above in table 17, except the annealing temperature was changed to 69 °C

2.12 Purification of PCR products

The PCR product was purified using a Roche™ high pure PCR purification kit. This kit contains three buffer solutions: binding buffer, washing buffer and elution buffer, a high pure column filter tube and collection tube. For purification, PCR product was joined to make up the volume to 100µL. PCR product was mixed with 500 µL of binding buffer, mixed well. After mixing, the solution was transferred to the high pure purification filter column and centrifuged at 13000 rpm for 1 minute. After centrifugation, the DNA was washed twice with wash buffer and purified PCR product was eluted to clean 1.5 mL centrifuge tube with 50-100 µL of elution buffer. The purified PCR can be used for the ligation / fusion PCR reaction.

2.13 Restriction digest of DNA (RD)

Restriction digestion is a process of splicing a DNA molecule into small DNA pieces using restriction endonucleases known as restriction enzymes (RE). The sensitivity of the restriction digest depends on enzyme activity and the buffer used for the digestion of the DNA molecule. Restriction digest is a widely used technique in recombinant DNA technology. Restriction digest reactions were set up by adding the PCR product/plasmid DNA with restriction enzyme in an appropriate buffer solution. The entire reaction was incubated at 37°C overnight. After digestion, the activity of the enzyme diminished via heating the reaction at 65-80°C only when

specified. The digested samples were visualised through agarose gel electrophoresis. See table 2.17 for protocol.

Table 2. 16 : Restriction digest protocol

	Vector with both enzyme	Vector with the only enzyme I	Vector with only enzyme II	Only vector
Reaction volume	200	20	20	20
Plasmid DNA	40	4	4	4
10 X buffer*	20	2	2	2
Enzyme I	3	0.3	0	0
Enzyme II	3	0	0.3	0
ddH_20	134	13.7	13.7	14

*NEB/CUT SMART buffer was used according to the requirement of the enzyme.

** double digest only performed for the enzymes with the same buffer requirement.

The presence of digested plasmid was confirmed via agarose gel electrophoresis. (0.8% agarose)

2.14 Purification of plasmid DNA vector

The digested vector was cleaned using zymoclean™ gel DNA recovery kit. This cleaning kit contains three buffer solutions: ABD buffer, DNA wash buffer and DNA elution buffer. To a 200 µL digested plasmid/vector 600 µL of ABD buffer was added and incubated for 15 minutes at 37 ℃. After incubation, the solution was transferred to a zymo-spin™ column with collection tube and centrifuged for 30-60 seconds. On centrifugation, solution flow-through was discarded and washed with 200 µL DNA washing buffer for 30 seconds. Wash was repeated. Clean vector was eluted with DNA elution buffer in 1.5 mL centrifuge tube. This purified clean vector was used for ligation reaction.

2.15 Agarose gel electrophoresis (AGE)

Agarose gel electrophoresis is a molecular technique used to separate a mixed population of macromolecules such as DNA. The separation of DNA fragments is dependent on the size of the DNA fragment and pore size of the agarose gel. The higher the gel percentage, the better the separation of the bands. The agarose was dissolved in 1x TAE buffer and heated in a microwave until the agarose is completely dissolved and cooled to 50℃. 10 µL of ethidium bromide (10 µg/µL) was added to the agarose and the solution poured into a casting tray and combs were placed to created sample wells. On cooling, the gel was placed in the electrophoresis apparatus and combs were removed carefully. The gel was totally submerged in TAE buffer. The DNA sample was prepared. To prepare the DNA sample, 5 µL of DNA sample was mixed with 1 µL 6 x gel loading dye and samples were loaded onto gel and a 2 log DNA (0.1-10 kb, New England biolab) ladder was used as a marker or standard reference. After loading the sample, the gel was run at a constant voltage of 100-125 Volt for 40 minutes. DNA bands were visualised through an ultraviolet transilluminator (biorad) and the images were captured and analysed using Quantity One software.

I. 1% agarose gel

1% agarose gel was prepared by dissolving 0.5 grams of agarose powder in 50 ml of 1X tris acetate EDTA buffer. A visualising agent, 5 µL of ethidium bromide (EtBr) was added to the gel and poured into the gel apparatus.

II. 0.8 % agarose gel

0.8% agarose gel was prepared by dissolving 0.4 grams of agarose powder in 50 ml of 1X tris acetated EDTA buffer. A visualising agent, ethidium bromide (Etbr) was added to the gel and poured into the gel apparatus.

III. 50 X Tris Acetate EDTA (TAE)

Tris (FORMEDIUM™)	276 g
Acetic acid	57.1 mL

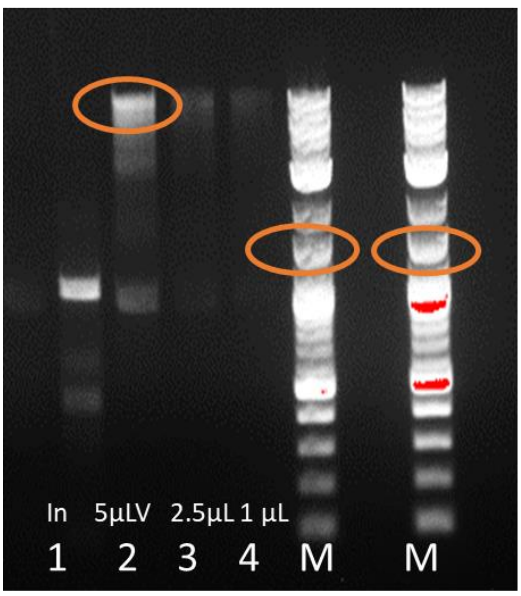
EDTA (Labsaver)	18.6 g
water	Added up to 1 L

IV. 6 X DNA loading dye

Bromophenol blue	0.25 mg
Glycerol	3 mL
Water	7 mL

2.16 Estimation of vector through agarose gel electrophoresis

The concentration of the vector/ PCR amplicon and plasmid DNA was determined via loading the variable amounts of DNA sample (1 µL, 2.5 µL and 5 µL) onto 0.8 % agarose gel along with 5 µL of 2 log DNA (5µl loaded) * ladder for 40 minutes. The band intensity of the loaded sample was determined via comparing with the 2 log DNA ladder. The approximately matched bands were used to calculate the amount of vector to be added to the ligation/ random mutation PCR reaction. The calculation is shown as follows.



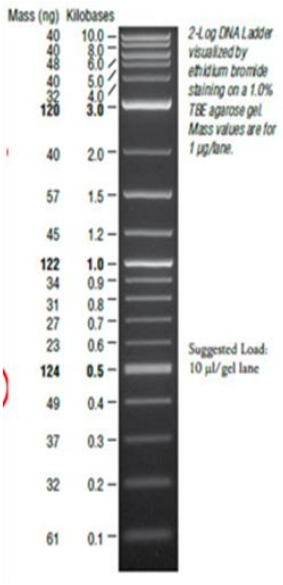


Figure 2. 1 Estimation of vector by agarose gel electrophoresis

The intensity of lane 2 DNA was compared with 2 log DNA marker and the amount of DNA was calculated. See below for sample calculation.

*5 µL loaded vector sample resembled 62 ng of the 2 log DNA ladder when compared. The amount of DNA in 1 µL of sample was calculated as follows

(Amount of DNA in 5 µL of sample × 1) ÷ Amount of sample loaded

In this case,

Amount of DNA in 5 µL of sample was 62 ng

Amount of sample loaded was 5 µL

Therefore, (Amount of DNA in 5 µL of sample × 1) ÷ Amount of sample loaded

$$(62\times1) \div 5 = 12.5 \text{ ng of DNA in } 1\mu L \text{ of sample}$$

To calculate 20 ng of vector,

20 ng ÷ the amount of DNA in 1µL of sample

In this case,

20 ng ÷ 12.5 = 1.6 µL of the vector was added to the ligation mixture

Similarly, the amount of DNA needed for random mutagenesis study was calculated.

2.17 Plasmid isolation by miniprep.

The plasmid was isolated from *E. coli* culture through the Roche™ miniprep isolation kit. The 4 mL overnight culture was centrifuged at 1500 rpm for 10 minutes. After this, the centrifugation pallet was resuspended in 250 µL suspension buffer and 250 µL lysis buffer and mixed gently. After mixing it was incubated for 5 min at room temperature. After incubation, 350 µL of binding buffer was added and mixed gently and incubated on ice for 5 min. After incubation the culture was centrifuged at 10,000 rpm for 10 minutes. After centrifugation, the supernatant was transferred to a column and centrifuged at max speed for 1 min. Flow-through was discarded, and the filter tube was washed with 500 µL of wash buffer I and centrifuged at

10,000 rpm for 1 min. Flow-through was discarded and the filter tube was washed with wash buffer II. The wash solution was spun down at 10,000 rpm for 1 minute. An extra spin was given to remove residues of the wash buffer. After spinning, elution buffer was added and spun down at 10,000 rpm for 1 min, and the purified plasmid was extracted. Agarose gel electrophoresis confirmed the presence of plasmid.

A. Composition of suspension Buffer

- 25 ml
- 50 mM Tris-HCl and 10 mM EDTA,
- pH 8.0 (+25° C)

B. RNase

- 2.5 mg • dry powder was dissolved in Suspension Buffer

C. Composition of lysis Buffer

- 25 ml
- 0.2 M NaOH and 1% SDS

D. Composition of binding Buffer

- 25 mL
- 4 M guanidine hydrochloride
- 0.5 M potassium acetate, pH 4.2

E. Composition of wash Buffer I

- 33 mL; add 20 mL absolute ethanol
- 5 M guanidine hydrochloride,
- 20 mM Tris-HCl, pH 6.6 (+25 °C) (final concentrations after addition of ethanol)

F. Composition of wash Buffer II

- 10 mL; add 40 mL absolute ethanol
- 20 mM NaCl, 2 mM Tris-HCl, pH 7.5 (+25 °C) (final concentrations after addition of ethanol)

G. Composition of elution Buffer

- 40 mL
- 10 mM Tris-HCl, pH 8.5 (+25 °C)

2.18 DNA Ligation

Preparation of the ligation process involves the generation of an insert and vector that is obtained through restriction digest (a protocol discussed previously). The amount of vector is calculated as described in methodology section 2.15. After determining vector concentration, the following ligation reaction was set up as mentioned below (Anders H. Lund, Morgens Duch, Finn Skou Pedersen, 1996).

Table 2. 17 . Ligation reaction set-up, in a volume of 8 µL

Tube	Double digested Plasmid	Insert in µL Double digested PCR
1	20 ng	60 ng
2	20 ng	6 ng
3	20 ng	2 ng
4	20 ng	-
5	-	1
6	-	-

After setting up the ligation reaction as mentioned in the table 2.18, the mixture was heated at 45 °C for 10 minutes and then immediately cooled down to 4 °C. In the meantime, one part of ligation buffer and one part of T_4 DNA ligase enzyme 2 units /µL (1:1 ratio) were added to each tube of the ligation reaction and mixed well through vertexing. On the PCR machine, the ligation protocol was set up and allowed to run overnight (see table 2.19). After overnight ligation, the mixture was heated to 65 °C. 1-2 µL was used for *E. coli* transformation (electrocompetent cells).

Table 2. 18 . Ligation protocol

Step	Temperature in °C	Duration	Cycles
to allow ligation reaction to occur	30 °C	20 seconds	999
to allow sticky ends to hybridise with each other	10 °C	20 seconds	
Hold	4 °C	∞	

2.19 Random mutagenesis of DNA

In this PCR reaction, the mutations were introduced through the error-prone PCR method, using mutazyme II from (genemorph kit Agilent ™). Mutazyme II™ is a mixture of novel DNA Taq polymerase and mutazymeI (Genemorph II™). To accumulate more mutations, the initial target DNA was adjusted to a low concentration with a high number of PCR cycles. With each cycle of PCR amplification, more mutations accumulate in the DNA. The higher the number of PCR cycles, the more mutations in the amplicon. Conversely, a lower PCR mutation is achieved via adding a high concentration of target DNA with a low amplification number.

The initial amount of target DNA was calculated by the Nanodrop spectrophotometer method or loading the sample against a 1 kb ladder on an agarose gel electrophoresis gel (method discussed earlier in section 2.15)

To set up high-frequency amplification, a master mix (50 µL) was prepared (dNTPs, primers, amplification buffer and polymerase) and then 9 µL of the master mix was distributed amongst five different vials, and the remaining 5 µL of the master mix was used as a negative control for the amplification reaction. The amplification reaction was set up as follows

Table 2. 19 . A standard PCR reaction mix

Component	Volume
PCR-grade water	Up to 10 µL
10 X Mutazyme buffer	1 µL
10 mM dNTP Mix	0.2 µL
10 µM Forward primer	0.05 µL
10 µM Reverse primer	0.05 µL
2.5 U/µl Mutazyme II polymerase	0.01 µL
Target DNA	10-125 ng/ µL

Table 2. 20 A cycling protocol for random mutagenesis PCR

Step	Temperature in °C	Duration	Cycles
Initial denaturation	95 °C	3 minutes	1
Denaturation	95 °C	30 seconds	35
Annealing	69 °C	30 seconds	
Extension	72 °C	60 seconds	
Final Extension	72 °C	10 minutes	1
Hold	4 °C	∞	

After amplification, 5 µL of amplified sample was loaded on a 1.3% agarose gel along with a 1 kb ladder. The concentration of the amplified product was estimated through AGE as mentioned earlier. After determining the concentration of the amplified product, the amplicon was diluted to the desired concentration to set up the next random mutagenesis reaction until the desired amount of mutation was introduced in the target DNA.

2.20 Next-generation sequencing (NGS)

Samples for sequencing were prepared according to the instructions of the company performing the sequencing reaction (Macrogen). After the generation of the sequence, the sequence was aligned through BLAST (basic local alignment search tool), to determine whether the sequence was as expected.

Chapter 3 Results

This thesis aimed to establish an assay that allows the identification of amino acids involved in the Gcn1-Gcn2 interaction. The general idea behind this study was to establish a library of randomly mutagenised RWDBD domains through random mutagenesis PCR. This library will then be screened for mutated RWDBD domains that have lost the ability to bind to Gcn2, through phenotypic enrichment and selection. In this study, the goal was to optimise induvial steps of this assay which are presented in the following sections.

3.1 Elimination of truncated RWDBD domains from the library of mutated RWDBD domains.

While introducing mutations in the Gcn2 binding region (RWDBD domain), it can occur that nonsense mutations may be introduced in the RWDBD domain. The RWDBD domains with a nonsense mutation will lead to truncated RWDBD domains which have lost the ability to bind to Gcn2 and will not be helpful in identifying the amino acids involved in Gcn1-Gcn2 interaction. Therefore, we aimed to design a mechanism to remove these truncated RWDBD domains from the pool of mutated domains. The mechanism used in our study was to place a *TRP1* gene downstream of the RWDBD domain. The placement of Trp1 after the C-terminal of RWDBD allows us to select the RWDBD domain only if it contains a sense mutation, allowing translation of Trp1, conferring the prototrophy for amino acid tryptophan. *TRP1* encodes the enzyme phosphoribosyl anthranilate isomerase which is involved in the tryptophan biosynthesis.

Next is given an outline of the construct used in this study. The RWDBD domain is under control of a galactose inducible promotor. Therefore, the RWDBD domain is only expressed when the medium is supplemented with galactose. A glutathione-s-transferase (GST) epitope tag is located upstream of the N-terminus of the RWDBD (see Figure 3.1). This GST epitope tag can be used for affinity purification, or for detecting the protein construct in western blot analysis. As described above, the C-terminal of RWDBD is fused to Trp1. Furthermore, by performing the western blot we can confirm the presence of a full-length RWDBD domain, via probing for the myc tag which was placed after Trp1 (see figure 3.1).

Figure 3. 1 Schematic representation of the RWDBD domain construct

The construct is under control of a galactose inducible promoter and expressed only when galactose is provided as a carbon source. GST is used as an epitope tag. Trp1 confers prototrophy to tryptophan when expressed. The presence of myc confirms full-length expression of the construct.

The following section describes how the presence of Trp1 will help to eliminate truncated RWDBD domains from the library of mutated RWDBD domains in detail. The presence of a nonsense mutation (denoted as # in Figure 3.2d) in the RWDBD domain, will lead to the premature termination of translation (see Figure 3.2d). As a result, the *TPR1* gene is not translated, and the strain remains auxotrophic for the amino acid tryptophan, whereas, the mutated RWDBD domain, without a nonsense mutation (denoted as * in Figure 3.2c), allows the *TRP1* gene translation, conferring prototrophy for the amino acid tryptophan (see Figure 3.2c). This phenotypic advantage aids in the selection of strains expressing mutated full-length RWDBD domains. In conclusion, this procedure allows us to eliminate the RWDBD domains with nonsense mutations by acquiring tryptophan prototrophy.

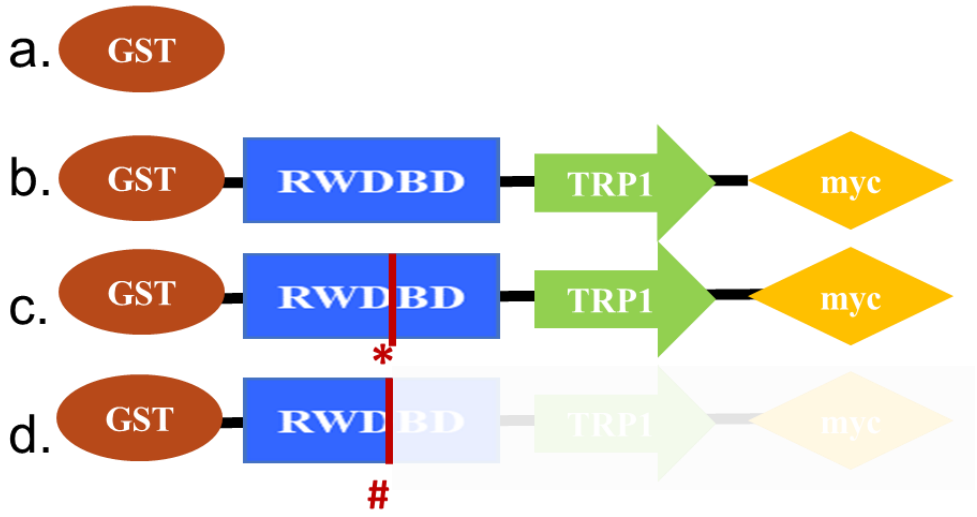


Figure 3. 2 Significances of Trp1 in the elimination of truncated RWDBD domains from the mutated RWDBD domain library.

a. When a yeast cell only expresses GST, it is auxotrophic for tryptophan. **b** When the yeast cell expresses the wildtype RWDBD (absence of mutations) fused to Trp1, the strain will be prototrophic for the amino acid tryptophan. **c.** When the yeast cell expresses a RWDBD domain containing a sense mutation (denoted by *) fused to Trp1, this construct still renders the strain prototrophic for the amino acid tryptophan. **d**. A nonsense mutation in the RWDBD (denoted as #) generates a premature stop codon leading to the translation of a truncated protein that lacks Trp1. Consequently, the strain is not tryptophan prototrophic and renders cells unable to grow in the absence of tryptophan.

3.1.1 Trp1 is functional when fused with RWDBD under a galactose inducible promoter

As discussed in the section above, we have fused our RWDBD domain with Trp1. Now it is essential to test the enzymatic function and stability of Trp1. The constructs were assessed via a semiquantitative growth assay, as outlined in section 2.10, “semiquantitative growth assay”, of the materials and methods section. In this assay, a yeast WT strain H1511 was transformed with plasmids expressing either only GST, GST-RWDBD-*TRP1*-myc, GST-RWDBD ^{R2259A}-*TRP1*-myc or control plasmid (confers tryptophan prototrophy). R2259A is a mutation in RWDBD, which is known to weaken the Gcn1-Gcn2 interaction. Two representative colonies of each transformant were selected and grown until saturation in medium containing amino acid isoleucine, leucine, valine, and tryptophan (ILVW) for 48 hours at 30℃ with shaking.

Five µl of 10-fold serially diluted culture (up to 1:10000 dilution) of these strains were transferred onto a different solid medium. One type of medium was a synthetic defined medium (SD) containing glucose with amino acid leucine, valine, isoleucine, and tryptophan (SGluWILV). The presence of glucose in this medium serves as a carbon source. The other solid medium was a synthetic defined medium (SD) with galactose and amino acid leucine, valine, isoleucine, and tryptophan (SGalWILV). The galactose in the medium is required for the induction of construct. SGal medium lacking tryptophan (SGalILV) was used to determine the prototrophic nature of the strains. The growth was monitored and recorded over three to four weeks. All growth was carried out in a 30 ^{o}C incubator.

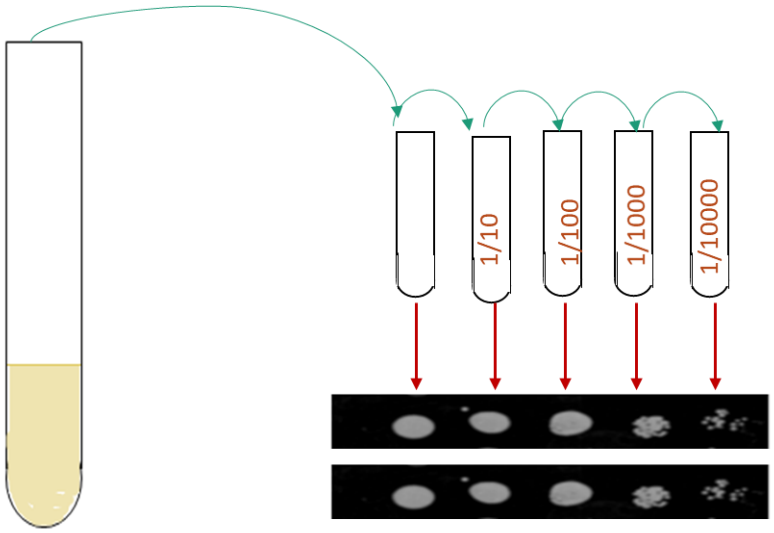


Figure 3. 3 Serial dilutions for semiquantitative growth assay.

The overnight grown culture was 10-fold serially diluted and 5 µL of each dilution spotted on solid medium.

On observation, the yeast WT strain H1511 expressing only GST (top two rows on the plate, (see Figure 3.4) failed to grow on the plate lacking tryptophan (SGal without tryptophan, see Figure 3.4). On the other hand, the WT strain expressing only GST could grow on the plate with tryptophan (SGluWILV, SGalWILV). This suggests, the yeast strains expressing only GST were auxotrophic for tryptophan, as expected (see Figure 3.4). Furthermore, the yeast WT strain H1511 expressing GST-RWDBD-*TRP1*-myc and GST-RWDBD ^{R2259A}-*TRP1*-myc were able to grow on galactose medium lacking tryptophan as seen in Figure 3.4. The latter suggests that the Trp1 is functional and confers prototrophy for tryptophan, indicating that the strains

are auxotrophic for Trp1 when grown on glucose containing medium and prototrophic when grown on galactose containing medium.

In conclusion, when Trp1 is fused with RWDBD, it is still capable of conferring tryptophan prototrophy.

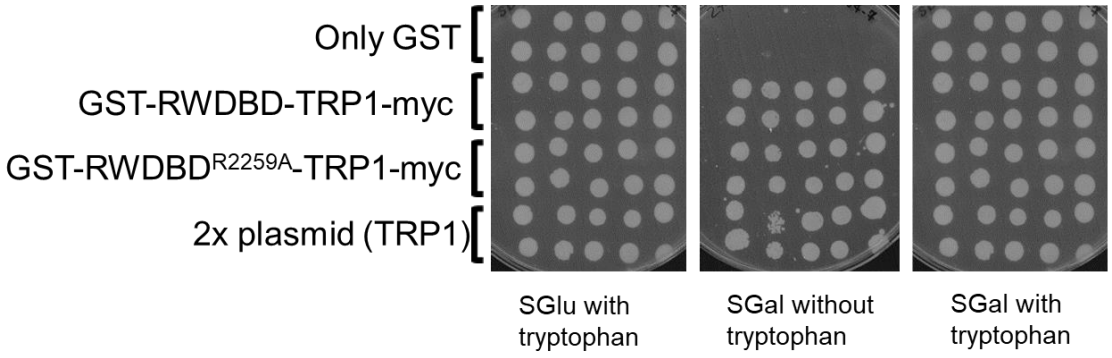


Figure 3. 4 Trp1 confers tryptophan prototrophy when expressed on galactose medium in the presence of RWDBD**.**

Ten-fold serially diluted transformant culture was dotted on solid medium supplemented with galactose or glucose as a carbon source. The strains expressing *TRP1* gene could grow on medium with and without tryptophan. GST alone was used as a negative control and as expected no growths were seen on the SGal plate without tryptophan for only GST. Furthermore, yeast expressing *TRP1* gene could grow in the absence of tryptophan.

3.1.2 The RWDBD is functional when fused to Trp1.

As mentioned in the "1.6 Gcn1-Gcn2 interaction" of the introduction, we know that for Gcn2 to be activated at all, it needs to interact with Gcn1. The interaction between Gcn1 and Gcn2 is governed by the RWD domain of Gcn2 and the RWDBD domain of Gcn1(Hinnebusch, 2000).

From earlier findings, we confirmed that the enzymatic function of Trp1 remains unaffected when fused to RWDBD. Here we aim to test whether the fusion of Trp1 to the RWDBD domain affects its ability to disrupt the Gcn1-Gcn2 interaction in starvation conditions via semiquantitative growth assays. The ability of yeast strains to grow under starvation conditions was analysed by inducing starvation for the amino acid histidine by 3 AT. If cells can activate Gcn2, then they are able to overcome starvation and grow on a starvation medium. If they are

unable to activate Gcn2 – for example by RWDBD disrupting Gcn1-Gcn2 interaction, they cannot grow on a 3 AT medium.

In this assay, we used yeast wildtype strain H1511 individually transformed with plasmids expressing from a galactose inducible promotor GST alone, RWDBD fused to Trp1(GST-RWDBD-TRP1-myc), GST-RWDBD^{R2259A}-TRP1-myc, RWDBD domain without Trp1 (GST-RWDBD-myc), GST-RWDBD^{R2259A}-myc, and strain expressing *gcn1*Δ was used. The *gcn1*Δ strain was used as a control to ensure that 3 AT did cause starvation. Two independent colonies of each transformed strain were selected and grown till saturation in a medium containing amino acids isoleucine, leucine, valine, and tryptophan (ILVW) for 48 hours at 30℃ with shaking. A semiquantitative growth assay was performed as discussed above.

The ability of these strains to grow under starvation conditions was analysed by inducing starvation for amino acid histidine by 3 AT, an inhibitor of the histidine biosynthesis pathway. The starvation response of these strains was monitored for their ability to grow under starvation conditions. The intensity of the starvation response can be monitored by increasing the concentration of 3 AT (starting from 10 mM to 120 mM of 3 AT) The growth was monitored and documented over three to four weeks or until no change in growth was observed. All growth was carried out in a 30 ^{o}C incubator.

We noticed that the H1511 strain expressing only GST could grow on all plates including starvation plates, whereas, the strain expressing GST-RWDBD-TRP1-myc failed to grow on 3 AT plates indicating that they are 3 AT sensitive (see Figure 3.5 10 mM 3 AT, 60 mM 3 AT). This suggests that the Gcn1-Gcn2 interaction was disrupted by overexpressed RWDBD. The overexpressed RWDBD domain binds to Gcn2, and, as a result, the yeast strain failed to activate the Gcn2 response and could not grow on the starvation medium, indicating the function of RWDBD domain is unaffected when fused to Trp1. Furthermore, when strains expressing GST-RWDBD-TRP1-myc were compared with strains expressing GST-RWDBD-myc, we showed a similar response. However, strains expressing GST-RWDBD-TRP1-myc showed increased sensitivity towards 3 AT (see Figure 3.5, 3 AT plates).

As expected, the strain expressing GST-RWDBD^{R2259A}-TRP1-myc and GST-RWDBD^{R2259A}-myc could grow on a SGalWILV medium supplemented with 60 mM 3 AT. This suggests that the overexpressed RWDBD domains have lost the ability to bind with Gcn2 due to the presence of mutations. Consequently, the strain can activate Gcn2 to overcome the histidine starvation

and grow. This suggests the function of the mutated RWDBD domain remains unaffected by Trp1 when fused to a c-terminal domain.

The Gcn1 deletion strain (*gcn*1Δ) was used to confirm starvation induced by 3AT. From the above observations, we can conclude that the presence of Trp1 does not affect the function of overexpressed RWDBD domains.

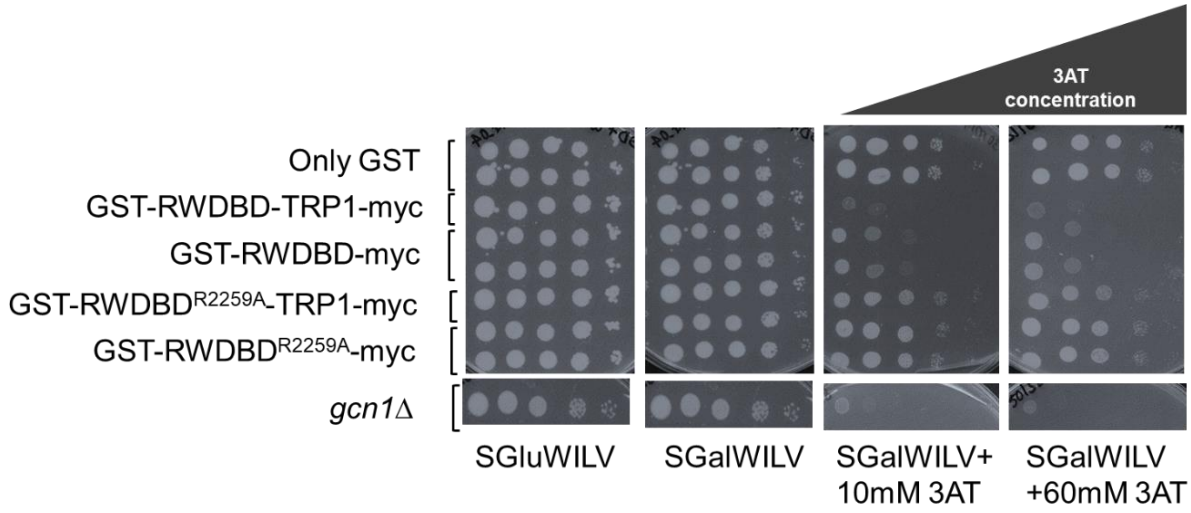


Figure 3. 5 Overexpressed RWDBD domain is functional when fused with Trp1.

5 µL of ten-fold serially diluted overnight cultures of yeast strains expressing the indicated constructs were transferred onto solid media containing 3 AT or not. On incubation we found that the strain expressing GST-RWDBD-TRP1-myc showed reduced growth on 3 AT plates, suggesting that it could disrupt the Gcn1-Gcn2 interaction, and that the RWDBD function is unaffected when fused to Trp1.

3.2 Determining the 3AT concentration that causes maximum growth discrepancy between yeast strains unable to activate Gcn2, and those able to active Gcn2.

In this thesis, we aim to establish a procedure to comprehensively identify additional amino acids that are essential for the Gcn1-Gcn2 interaction. One way to do this is by altering amino acid(s) in the RWDBD domain through random mutagenesis and then enriching the mutated RWDBDs that have lost the ability to bind to Gcn2, through competitive growth in starvation conditions.

For the competitive growth assay, we take advantage of the fact that strains unable to activate Gcn2 have reduced growth under starvation conditions. The more these cells starve, the more is their growth impaired, whereas the strains able to activate Gcn2 can overcome starvation and therefore are able to grow in the starvation medium. The Gcn2 function could be disrupted via abolishing the Gcn1-Gcn2 interaction in the yeast cell. The idea behind our approach is to overexpress the RWDBD, which would disrupt the Gcn1-Gcn2 interaction and impair Gcn2 activation. If amino acid(s) in the RWDBD are mutated that are important for Gcn2 binding, then this mutated RWDBD is not able to disrupt the Gcn1-Gcn2 interaction and the strain would be able to grow under starvation conditions. Using this approach, we aim to optimise the competitive growth under starvation conditions to enrich for strains containing mutated RWDBD unable to disrupt the Gcn1-Gcn2 interaction. To optimise the competitive growth condition, we first need to determine the minimal 3 AT concentration that leads to the maximal growth difference between strains able and unable to activate Gcn2.

3 AT used to induce starvation in the yeast cell for amino acid histidine. To discover this 3AT concentration, we scored the growth rate of a wildtype and a strain lacking Gcn1 and therefore unable to activate Gcn2.

To ascertain the growth rate under various 3 AT concentrations, an overnight culture of each, a wildtype yeast strain and a Gcn1 deletion strain, each expressing only GST from a galactose inducible promotor, was used inoculate the SGalWILV medium with differing concentrations of 3 AT (0.05 mM, 0.5 mM, 5 mM, 30 mM, 60 mM, 90 mM, and 120 mM). After inoculation, strains were incubated at 30 °C with continuous shaking, and the OD_{600} was measured every two hours. The growth patterns of both strains were evaluated by plotting the absorbances against the incubation time in a half-logarithmic fashion (see Figure 3.6, wildtype). In the

absence of 3 AT, the wildtype *gcn1*Δ strain showed a higher growth rate in the absence of starvation conditions compared to growth under starvation conditions. As the concentrations of 3AT were increased, the growth rate of the *gcn1*Δ *strain* decreased.

In the absence of 3 AT, the wildtype yeast strain was able to enter an exponential growth phase after 11 hours of incubation (see Figure 3.6, wildtype). However, the wildtype yeast strain, in media containing a 30-120 mM 3 AT, failed to enter the exponential growth phase after 11 hours (see Figure 3.6, wildtype). This suggested that these 3 AT concentrations were too high for any substantial growth to occur and that, despite the activation of Gcn2, the cells were unable to overcome starvation (see Figure 3.6, wildtype). In the 0.5 mM 3 AT the lag phase was very long, about 15 hours, until the strain was able to enter the exponential phase. At 0.05 mM 3 AT the growth behaviour was similar to that in medium lacking 3 AT, suggesting that at this 3 AT concentration the strain was able to quickly overcome starvation. In comparison, the yeast *gcn1Δ* strain was able to enter an exponential phase after 17 hours of incubation (see Figure 3.6, *gcn1*Δ). However, the medium supplemented with 30-120 mM 3 AT for *gcn1Δ* strains could not enter an exponential phase, even after a prolonged incubation time (see Figure 3.6 *gcn1*Δ*)*. At a concentration of 0.5 mM 3 AT, the Gcn1 deletion strain showed growth, but the lag phase was much longer than that of the wildtype strain.

In conclusion, a 3 AT concentration of 0.5 mM showed maximum growth discrepancy between yeast strains which can activate Gcn2 and were unable to activate Gcn2 in starved conditions

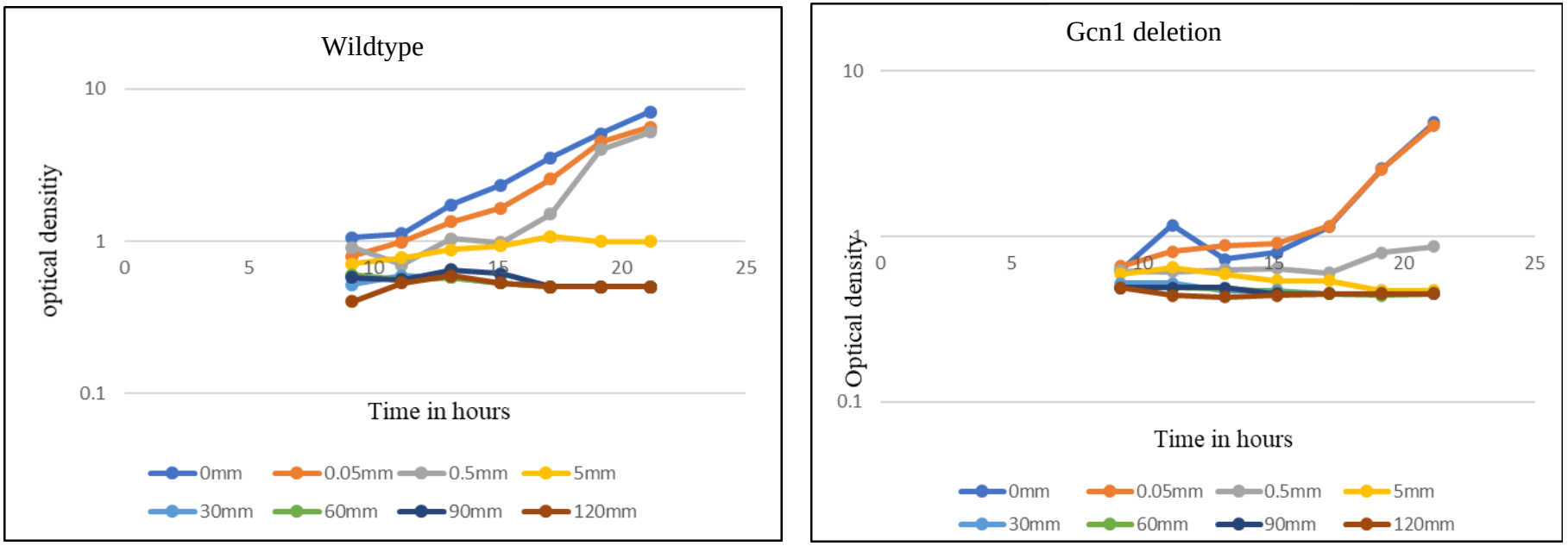


Figure 3. 6 Growth pattern of strains expressing wildtype and Gcn1 deletion under starvation conditions.

The overnight grown culture was inoculated in SGalWILV medium with different 3 AT concentrations and incubated for 24 hours at 30℃ with shaking. The growth of each strain was recorded over 24 hours and the OD_{600} was measured after every 2 hours. The graph was plotted for each strain. We noticed that as the concentration of the drug is increasing the growth of each strain is affected, and strains take longer to reach the exponential phase. All data points of each strain are listed in the appendix section.

3.2.1 Determining the minimal concentration of 3AT to enrich yeast strains expressing mutated RWDBDs which have lost the ability to bind Gcn2.

After determining the maximum concentration of 3 AT (in which wildtype yeast expressing only GST would grow but the *gcn1Δ* would not), the growth patterns of the yeast strains overexpressing RWDBDs were studied and growth assays were performed as mentioned in the above section. In this study, a yeast strain expressing GST-RWDBD-TRP1-myc overexpresses wildtype of RWDBD whereas, yeast strain expressing GST-RWDBD^{R2259A}-TRP1-myc overexpresses mutated RWDBDs. The R2259A mutation was used to represent the mutated RWDBDs.

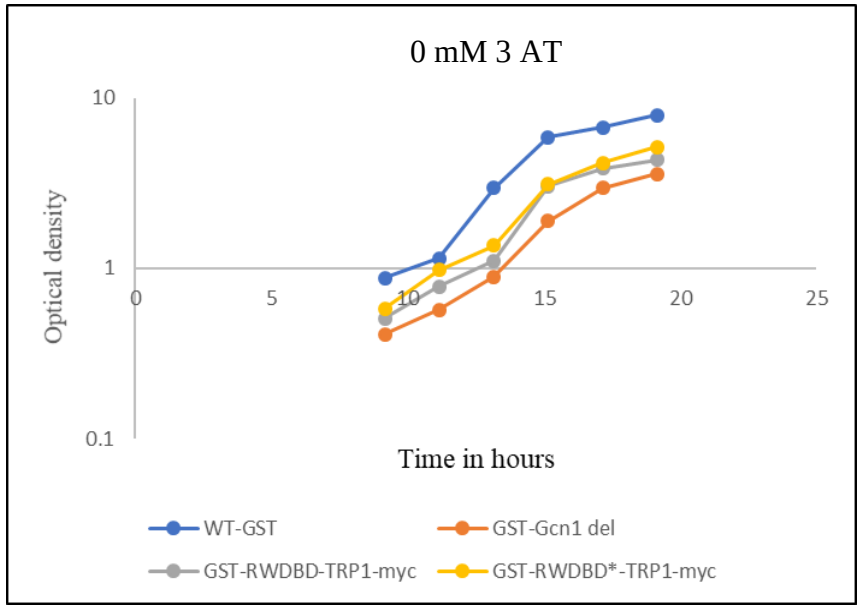
An overnight culture of each strain was used to inoculate the SGalWILV medium with differing concentrations of 3 AT (0.025 mM, 0.5 mM, and 1 mM). Here, we have used different concentrations of 3 AT to understand the growth patterns of each strain at minimum concentration (0.025 mM), medium concentration (0.5 mM) and higher 3 AT concentration (1 mM) and growth assay was carried out as mentioned above. The higher growth rate of strains indicates the absence of starvation conditions, whereas a reduced pattern of growth rate was taken as evidence that the strains had entered starvation conditions.

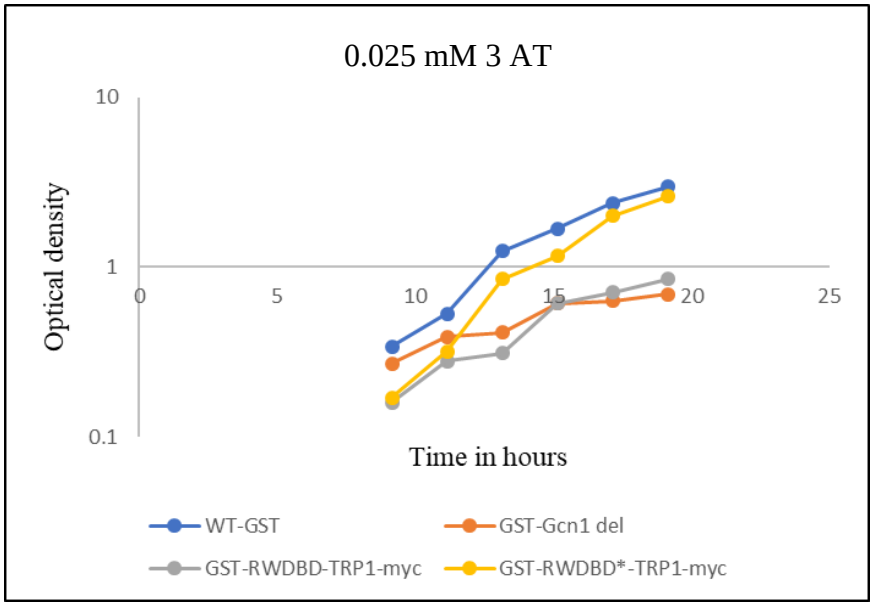
In our study, we found that the yeast strain expressing GST-RWDBD^{R2259A}-TRP1-myc showed a higher growth rate in non-starvation conditions and could enter an exponential growth phase after 11 hours of incubation (see Figure 3.7). As the incubation time progressed, the OD increased, whereas the strains supplemented with 1 mM 3 AT took longer (16 hours) to enter an exponential growth phase (see Figure 3.7).

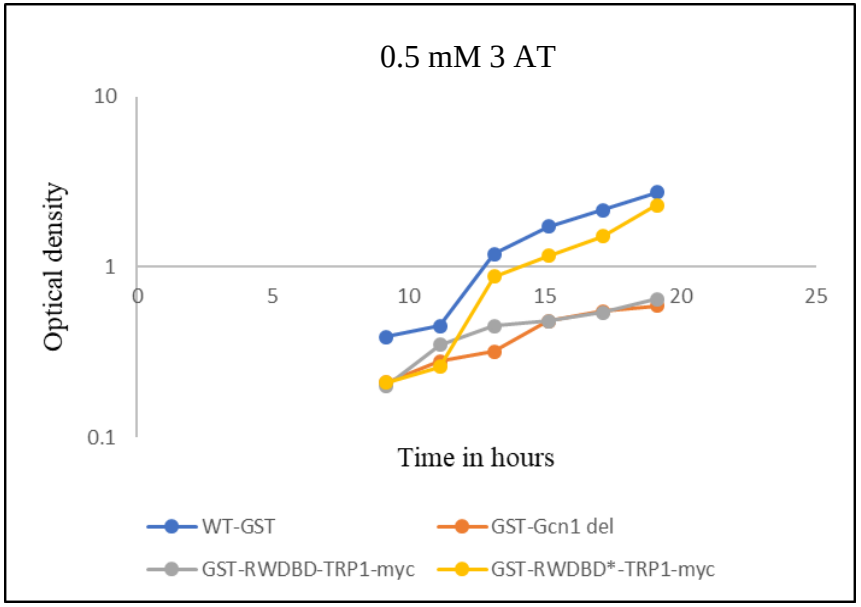
The yeast strain expressing GST-RWDBD-TRP1-myc showed a higher growth rate in the absence of 3 AT (non-starvation conditions) and entered an exponential phase after 11 hours of incubation, as expected (see Figure 3.7, 0 mM 3 AT). Furthermore, when this strain was supplemented with 0.025 mM and 0.5 mM and 1 mM of 3 AT, it exhibited slower growth and was unable to enter an exponential phase (see Figure 3.7), even when incubated for a longer time.

Additionally, when the growth pattern of strain expressing GST-RWDBD-TRP1-myc and GST-RWDBD ^{R2259A}-TRP1-myc were compared, we found that at 0.5 mM of 3 AT, showed maximum growth discrepancy between the strains. This suggests that 0.5 mM 3 AT concentrations can be used to induce starvation conditions in a competition assay.

To conclude, our observations of growth assay suggest that, at 0.5 mM 3 AT, the yeast strains expressing GST-RWDBD ^{R2259A}-TRP1-myc will be enriched during competitive growth.


0 mM 3 AT
Optical density
10
1
0.1
0
5
10
15
20
25
Time in hours
WT-GST
GST-Gcn1 del
GST-RWDBD-TRP1-myc
GST-RWDBD*-TRP1-myc


0.025 mM 3 AT
Optical density
10
1
0.1
0
5
10
15
20
25
Time in hours
WT-GST
GST-Gcn1 del
GST-RWDBD-TRP1-myc
GST-RWDBD*-TRP1-myc


0.5 mM 3 AT
Optical density
10
1
0.1
0
5
10
15
20
25
Time in hours
WT-GST
GST-Gcn1 del
GST-RWDBD-TRP1-myc
GST-RWDBD*-TRP1-myc

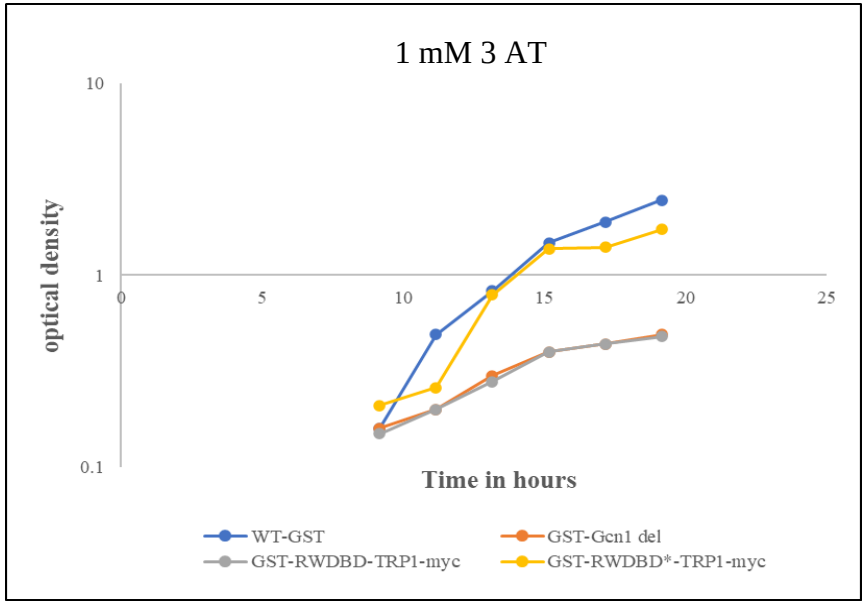

1 mM 3 AT
optical density
10
1
0.1
0
5
10
15
20
25
Time in hours
WT-GST
GST-Gcn1 del
GST-RWDBD-TRP1-myc
GST-RWDBD*-TRP1-myc

Figure 3. 7 Growth pattern of strains expressing GST-RWDBD-TRP1-myc, wildtype GST, GST-RWDBD^{R2259A}-TRP1-myc and GST-gcn1Δ in the starvation condition.

The overnight grown culture of a strain was inoculated in SGalWILV medium with different 3 AT concentrations and incubated for 24 hours at 30℃ with shaking. The growth of each strain was recorded over 24 hours, and the absorbances were measured after every 2 hours at 600 nm. The graph was plotted for each strain. We noticed that as the concentration of the drug increased, the growth of each strain was affected, and strains take longer to reach the exponential phase. All data points of each strain are listed in the appendix section.

3.3 An attempt to optimise competitive growth to enrich strains expressing GST-RWDBD^{R2259A}-TRP1-myc in starvation medium.

The competitive growth approach will take advantage of the fact that RWDBDs with the inability to bind Gcn2 would allow yeast cells to overcome starvation induced by 3 AT, and therefore will be able to grow (see Figure 3.8, a). While yeast strains expressing a mutated RWDBD domain capable of disrupting the Gcn1-Gcn2 interaction will allow yeast cells to grow on a starvation medium (see Figure 3.8, b). In this study, we aim to overexpress RWDBDs and select those who have lost the ability to bind to Gcn2, for example, when a yeast strain overexpresses mutated RWDBDs unable to bind to Gcn2 and it would not disrupt the Gcn1-Gcn2 interaction. Consequently, these yeast strains could overcome the starvation and be able to grow on a starvation medium (see Figure 3.8, c). In contrast, when yeast strains overexpress wildtype RWDBDs that can still bind to the Gcn2 binding region - and disrupt the interaction of Gcn1-Gcn2 - these yeast cells would be unable to overcome the starvation response and therefore cannot grow.

The goal of this chapter was to determine the time required to enrich yeast strains expressing mutated RWDBDs unable to bind to Gcn2. As a representative of such a mutated RWDBD we used RWDBD^{R2259A}, in form of the GST-RWDBD^{R2259A}-TRP1-myc construct. For the optimisation of the competitive growth, we needed an easy approach to differentiate between the strains expressing GST-RWDBD^{R2259A}-TRP1-myc and GST-RWDBD-TRP1-myc construct. For this we aimed to take advantage of a second selectable marker present in the backbone of one of the plasmids that conferred prototrophy to Leucine (*LEU2*), while the marker is absent in the other plasmid. The idea was that by simply growing aliquots on medium lacking and containing Leucine, we would be able to determine how many strains expressed the mutated RWDBD and how many the wildtype RWDBD. The available plasmids expressing GST-RWDBD^{R2259A}-TRP1-myc (pSG 50) and GST-RWDBD-TRP1-myc (pSG 52) both contained the *LEU2* marker conferring Leucine prototrophy. Thus, here the aim was to generate a plasmid (pAG 01) expressing the GST-RWDBD-TRP1-myc construct that lacked the *LEU2* marker in its backbone, meaning that this plasmid will not confer leucine prototrophy.

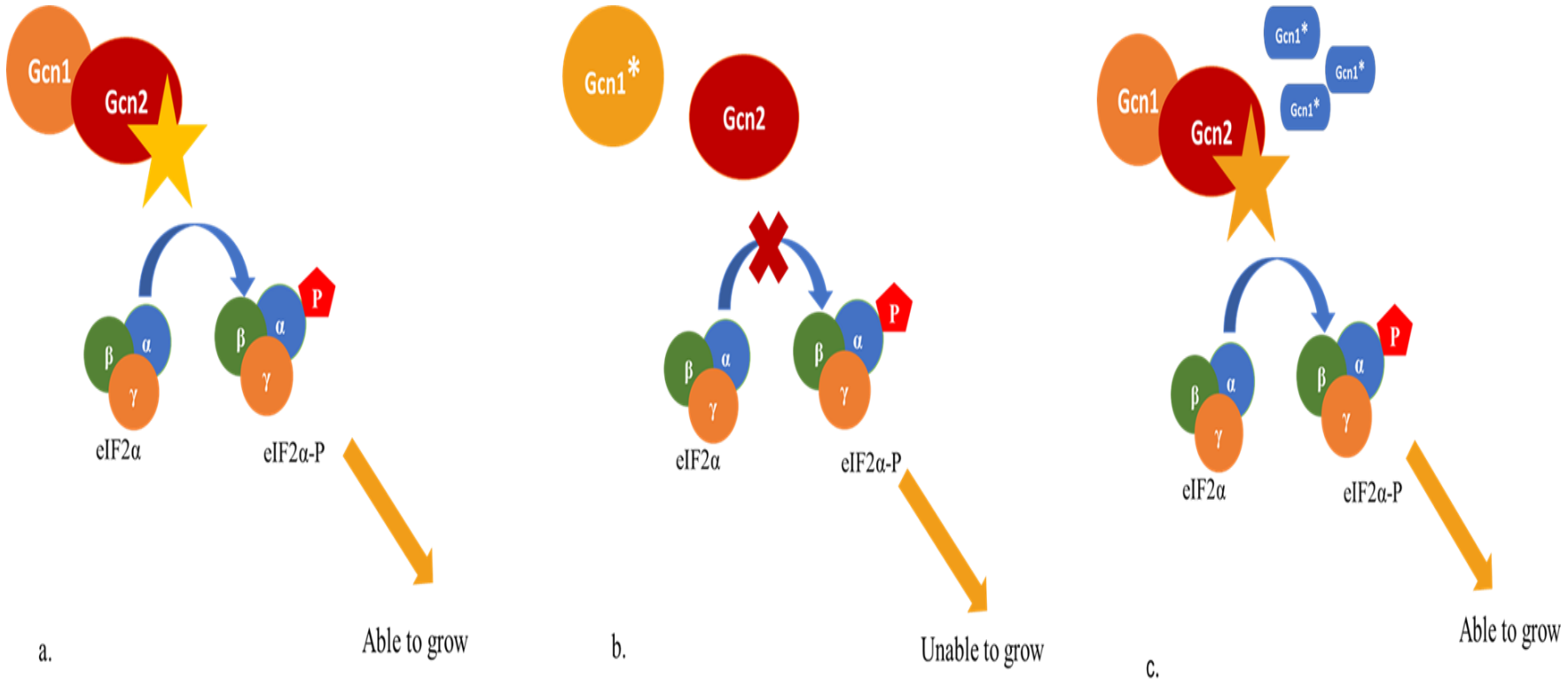


Figure 3. 8 An overview of Gcn2 function.

a. when in a yeast cell, Gcn1 can interact with Gcn2, it can grow on starvation medium **b.** When in a yeast cell Gcn1 is mutated, it cannot interact with Gcn2, and is unable to grow on starvation medium. **c** when in a yeast cell the mutated Gcn1 is overexpressed, the yeast cell can grow on starvation medium by activating Gcn2.

To construct a plasmid which lacked the *LEU*2, we used the plasmid pES125-1 containing the only GST tag but lacking the *LEU2* marker. This plasmid was cut open with restriction enzymes *SalI* (5'G/TCGAC3'), and *BglII* (5'A/GATCT3') and the double digest was performed as mentioned in section 2.13. These enzymes cut the plasmid at the multiple cloning sites. On completion of the restriction digestion, the enzymes were inactivated by incubating the reaction mixture at 65 °C for 20 minutes. The activity of each enzyme was determined by resolving an aliquot of the digested plasmids with electrophoresis through 0.8% agarose. As a control, the uncut plasmid was loaded along with a single and double digested plasmid. Molecular weight markers allowed an estimation of the size of the digested plasmid. (Figure 3.9, lane 5)

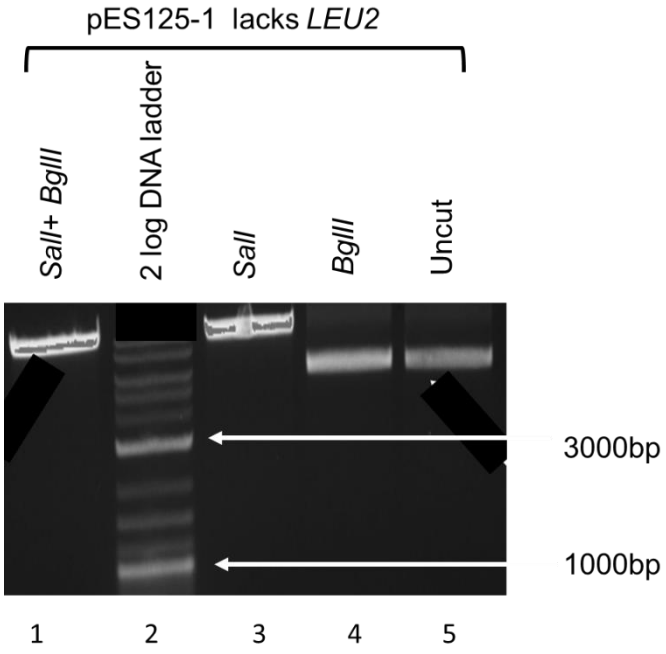


Figure 3. 9 Restriction digest of plasmid pES125-1.

Samples digested with the enzyme as indicated were resolved on a 0.8% agarose gel through electrophoresis. A single band was observed for double digested plasmid and *SalI* digested plasmid.

The plasmid digested by the *SalI* enzyme produced a single DNA band at 9300 bp indicating the plasmid was cut successfully by the *SalI* enzyme (Figure 3.9, lane 3). However, the plasmid

appeared to be unaffected by the exposure of *BglII* as the resulting band (Figure 3.9, lane 4) because it migrates similarly to that of the uncut plasmid (Figure 3.9, lane 5).

This would suggest that the plasmid in our double digest was only cut by the *SalI* enzyme and not by the *BglII* enzyme. The plasmid was again exposed to the *BglII* enzyme. Additionally, a small aliquot of plasmid was digested along with the double digest at 37 °C overnight as a control. Upon completion of digestion, an aliquot of double digest, single digest and uncut plasmid were resolved via electrophoresis through an 0.8% agarose gel.

On resolving, we saw that the plasmid was digested by the *BglII* enzyme as single bands as seen on the gel (Figure 3.10, lane 3). The DNA bands observed in lane 4 in Figure 3.10 represent the supercoil and relaxed structure of the uncut plasmid. On resolving we observed the entire digestion of the vector by *BglII*.

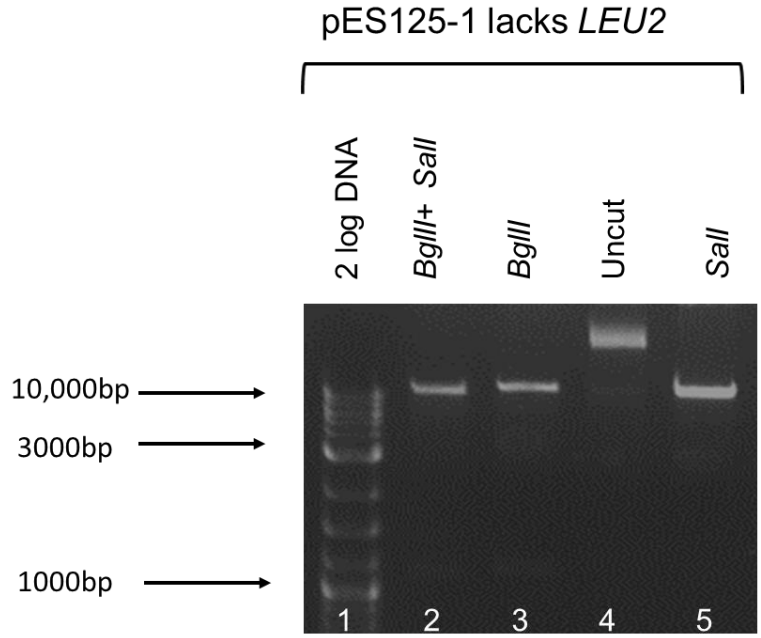


Figure 3. 10 Re-digestion of plasmid pES125-1 by BglII.

The DNA was digested with the enzyme as indicated and then an aliquot resolved on 0.8 % of agarose gel through electrophoresis.

The next step was to amplify a DNA fragment containing the coding sequence for the GST-RWDBD-TRP1-myc construct, using plasmid pES124-B-1 (pSG 52) as a template. For this PCR reaction, a varying amount of plasmid DNA was used for amplification. The amplification was carried out with primers ES 3275 and ES 3280 in the presence of *a Pfx* polymerase. The primers anneal to the GST-RWDBD-TRP1-myc fragment of the plasmid. The PCR amplicon

was resolved in a 1% agarose gel. A predominant single band was found at 1000 bp, corresponding to the expected 1000 bp size of the DNA fragment, suggesting that the PCR amplification was successful (Figure 3.11).

Following the digestion of the plasmid lacking the *LEU2* marker and the amplification of GST-RWDBD-TRP1-myc from the original pES124-B-1 plasmid, the linearized plasmid and the PCR amplicon were inserted into yeast cells via transformation. The yeast strains which could grow in absence of leucine were selected.

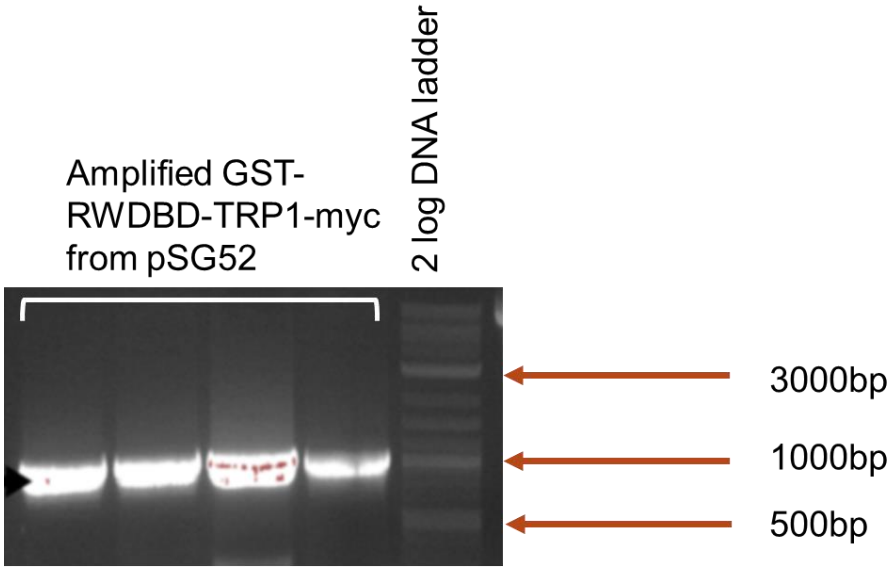


Figure 3. 11 PCR amplification of DNA coding for GST-RWDBD-TRP1-myc.

For PCR amplification plasmid pES124-B1 was used as template, the Pfx polymerase, and primers ES 3275 and ES 3280. The PCR reaction was resolved on 1.2 % of agarose gel through electrophoresis.

Furthermore, it is necessary to determine the ability of strains expressing pAG 01 which lacked the *LEU2 (LEU2*Δ*)* to disrupt the Gcn1-Gcn2 interaction when overexpressed under starvation conditions. This new construct from now on will be known as GST-RWDBD-*TRP1*-myc $^{leu2\Delta.}$

The yeast strain expressing GST-RWDBD-TRP1- myc $^{leu2\Delta}$ was tested for its ability to abrogate the Gcn1-Gcn2 interaction in starvation conditions by semi-quantitative growth assays. In this assay, the wildtype yeast H1511 strain expressing only GST, GST-RWDBD-TRP1-myc, GST-RWDBD-*TRP1*-myc $^{leu2\Delta}$, GST-RWDBD^{R2259A}-TRP1-myc, GST-RWDBD^{R2259A}-myc, and the yeast strain expressing GST-*gcn1*Δ were used. The yeast strain expressing GST- *gcn1*Δ was

used as a control to test whether 3AT starvation did occur. Two representative colonies of each transformant were selected and grown till saturation. These grown cultures were then used to perform a semi-quantitative growth assay as mentioned in section 2.10.

The growth pattern of each strain was observed, and we noticed that the wildtype yeast strain expressing only GST could grow on all plates including the starvation plates (see Figure 3.12), as expected, whereas the yeast strain expressing GST-RWDBD-TRP1-myc and yeast strain expressing GST-RWDBD-*TRP1*-myc $^{leu2\Delta}$ could not grow on the plate with 3 AT. This suggests that the strains expressing GST-RWDBD-*TRP1*-myc $^{leu2\Delta}$ could disrupt the Gcn1-Gcn2 interaction.

This suggests that the addition of the second selectable marker *LEU2*Δ does not affect the function of overexpressed RWDBD, can bind to the Gcn2 binding region, and is able to disrupt the Gcn1-Gcn2 interaction indicating strains are 3 AT sensitive (see Figure 3.12, 10 mM 3AT, 90 mM 3AT) as expected.

Furthermore, we also showed that the yeast strains expressing GST-RWDBD-*TRP1*-myc $^{leu2\Delta}$ could not grow on a plate lacking leucine (see Figure 3.12, SGlu with tryptophan). This suggests that the strain expressing the GST-RWDBD-*TRP1*-myc $^{leu2\Delta}$ plasmid is auxotrophic for leucine.

On the other hand, the yeast strain expressing GST-RWDBD^{R2259A}-myc and yeast strains GST-RWDBD^{R2259A}-TRP1-myc showed no sensitivity towards 3 AT, indicating they could grow on a starvation plate (see Figure 3.12, 10 mM 3 AT, 90 mM 3 AT) as expected.

The Gcn1 deletion strain (*gcn1*Δ) was used as a control to confirm that the drug 3AT induced the starvation condition. We found that this strain was unable to overcome starvation conditions when transferred onto the starvation medium, as expected. This confirms that 3AT and the concentrations used were sufficient to induce starvation for amino acid histidine in yeast (see Figure 3.12, Gcn1 deletion).

To ensure that the plasmid pAG 01 expressing GST-RWDBD-*TRP1*-myc $^{leu2\Delta}$ did not contain any unwanted mutations, it was sequenced. We found that the plasmid contained the desired sequence (see appendix Figure 5.8).

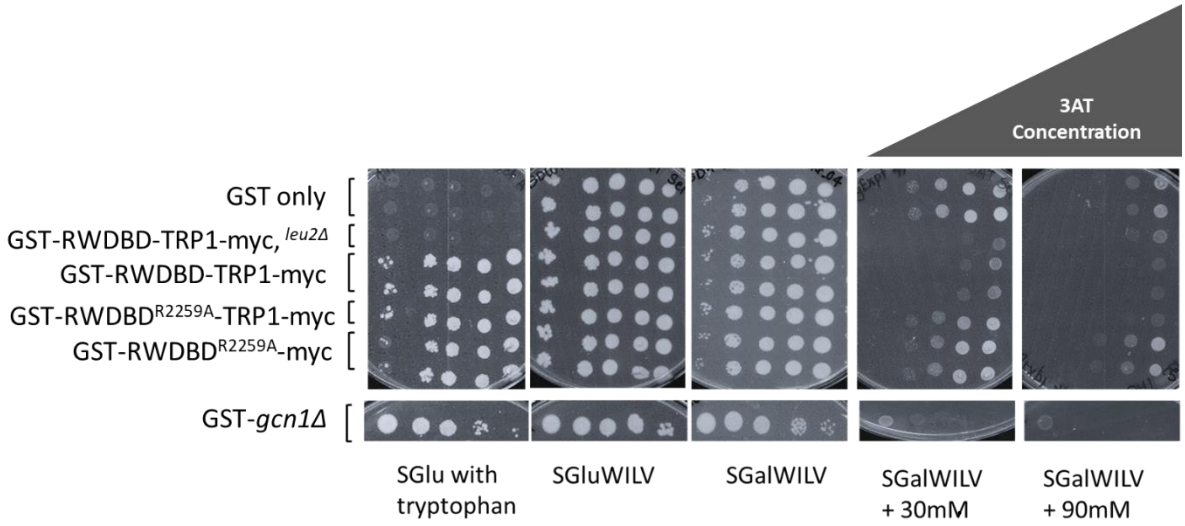


Figure 3. 12. Testing pAG01 plasmids ability to confer leucine auxotrophy.

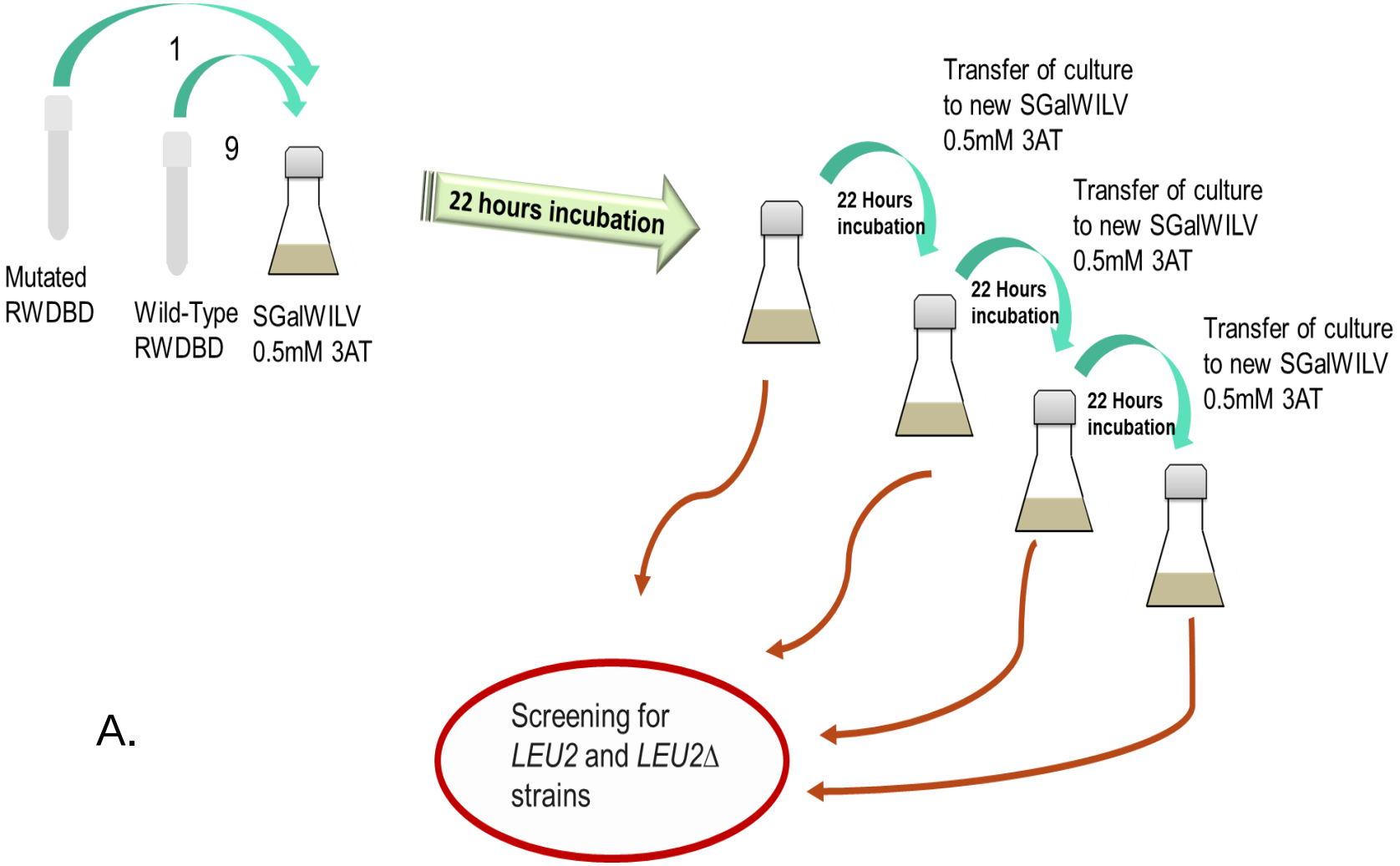
5 µL of ten-fold serially diluted overnight grown culture of yeast H1511 expressing the above constructs were transferred onto solid media as indicated. The yeast strain expressing GST-RWDBD-*TRP1*-myc $^{LEU2\Delta}$ plasmid could not grow on a plate lacking the leucine, which suggests that the strain is auxotrophic for leucine.

3.3.1 Enrichment of the strain expressing mutated RWDBDs that have lost their ability to bind to Gcn2 through competitive growth.

As mentioned in section 3.3, when the mutated RWDBDs are overexpressed in a yeast cell that could not bind to the Gcn2 binding region and could not disrupt the Gcn1-Gcn2 interaction, the yeast cells could grow on a starvation medium while the yeast strain overexpressing the wildtype RWDBDs could not grow on starvation medium. Using this principle, we aimed to optimise the competitive growth in starvation when RWDBDs are overexpressed.

The competitive growth was optimised using yeast strains expressing GST-RWDBD-TRP1-myc $^{LEU2\Delta}$ and GST-RWDBD^{R2259A}-TRP1-myc individually. Both strains were grown to saturation in a SGluWILV medium for 48 hours. To initiate the competitive growth, we used a starter culture with 0.5 optical density at 600 nm and the culture was inoculated to SGalWILV, 0.5 mM 3AT. The starter culture contained nine parts of yeast strains expressing GST-RWDBD-TRP1-myc $^{LEU2\Delta}$ and one part of yeast strains expressing GST-RWDBD^{R2259A}-TRP1-myc. The competitive growth was carried out as mentioned in section 2.9 (see Figure 3.13).

As described, the aim of competitive growth is to enrich the yeast strains expressing mutated RWDBDs unable to bind with Gcn2. For this an aliquot of the culture was taken after every 22-24 hours of competitive growth and reinoculated into fresh liquid medium supplemented with 3 AT (see Figure 3.13). Furthermore, to investigate the proportion of yeast strains in the culture expressing GST-RWDBD^{R2259A}-TRP1-myc, a tenfold serially diluted culture was plated onto SGluW (only tryptophan) and SGluWILV plates and incubated at 30 ℃ for 48 hours (see Figure 3.13). This entire process of re-inoculating culture into fresh medium was repeated until all the strains tested expressed GST-RWDBD^{R2259A}-TRP1-myc - which were prototrophic for Leucine.

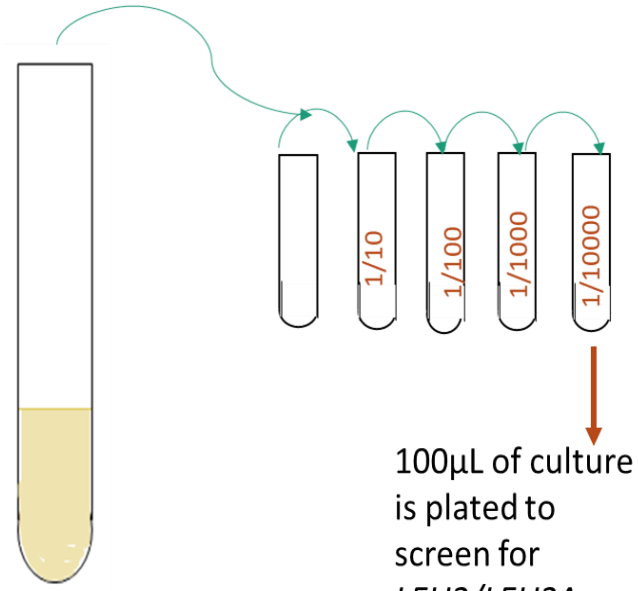

1
9
Mutated
RWDBD
Wild-Type
RWDBD
SGalWILV
0.5mM 3AT
22 hours incubation
Transfer of culture
to new SGalWILV
0.5mM 3AT
22 Hours
incubation
Transfer of culture
to new SGalWILV
0.5mM 3AT
22 Hours
incubation
Transfer of culture
to new SGalWILV
0.5mM 3AT
22 Hours
incubation
Screening for
LEU2 and LEU2Δ
strains

A.

B.

Screening for *LEU2* and *LEU2Δ* strains

a.



100µL of culture is plated to screen for *LEU2/LEU2Δ* strains

b.	If 10% cells are *LEUΔ*	If 90% cells are *LEUΔ*
 SD + W allow growth of *LEU* strains	 SD + W allow growth of *LEU* strains	 SD + W allow growth of *LEU* strains
 SD+WILV allow growth of *LEU/LEUΔ* strains	 SD+WILV allow growth of *LEU/LEUΔ* strains	 SD+WILV allow growth of *LEU/LEUΔ* strains

Figure 3. 13. Overview of competition assay.

A. The yeast strains expressing the GST-RWDBD-TRP1-myc construct and yeast strains expressing the GST-RWDBD^{R2259A}-TRP1-myc were mixed in a 9:1 ratio and incubated for 22 hours. Then, an aliquot of this culture was used to inoculate fresh SGalWILV medium with 0.5 mM 3AT. B. screening for *LEU2/LEU2*Δ strains a. Serially diluted aliquots of the initial culture were used for *LEU2*$^{+}$*/LEU2*Δ screening.

B. 1/10000 diluted culture was plated on SGlu with tryptophan and SGluWILV and incubated for a minimum of 48 hours, at 30°C. After 48 hours of incubation, the number of colonies was calculated for both plates, and the data were normalised to determine the ratio. See section 2.9 for normalisation of data.

Up to the time of 120 hours of incubation, we found that with increasing time of competitive growth, the number of *leu2*Δ strains decreased (see Figures 5.1 and 5.2, SGluW plate, 0.5 mM 3AT). This finding suggested that the strains expressing GST-RWDBD-TRP1-myc $^{LEU2\Delta}$ (wildtype RWDBD) could not overcome the starvation due to a disrupted Gcn1-Gcn2 interaction, and were impaired in growth, as expected. On the other hand, the number of *LEU2*$^{+}$ yeast strains were increased, as judged by growth tests on the SGluW plate. This suggested that the strains expressing GST-RWDBD^{R2259A}-TRP1-myc (mutated RWDBD) could overcome the starvation as the mutated RWDBDs allowed yeast strains to activate the Gcn2 starvation response and grow, as expected (see Table 3.1 and Figure 3.14). The above growth test of strains able to grow in the absence of leucine from now on will referred as the leucine prototrophy test.

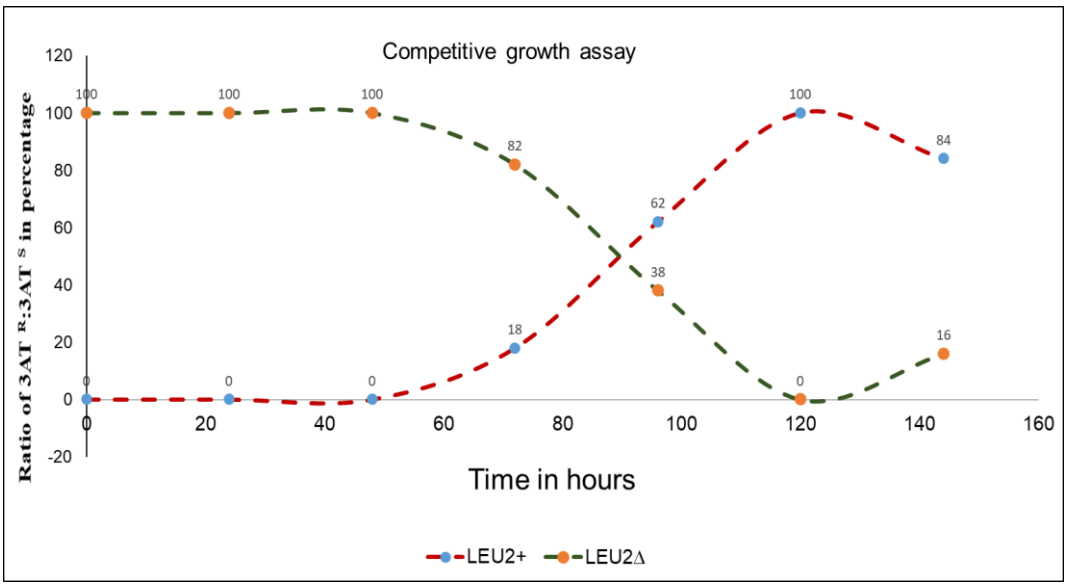


Figure 3. 14A. The ratio of *LEU2*$^{+}$ *to LEU2*Δ during competitive growth through the leucine prototrophy test.

The ratio of colonies was calculated for 100 µL of 1:10,000 (0.01µL) diluted culture was plated. As the incubation hours of competitive growth increased the yeast strain expressing *LEU2*Δ decreased, and the ratio of yeast strain expressing *LEU2*$^{+}$ increased. For obtained ratio and calculation see table 3.1

Table 3. 1 The ratio of *LEU2*$^{+}$ *to LEU2*Δ expressing strains during competitive growth using the leucine prototrophy test

Yeast strains expressing GST-RWDBD-TRP1-myc $^{LEU2\Delta}$ are auxotrophic for leucine. At the same time, yeast strains expressing *GST-RWDBD*^{R2259A}*-TRP1-myc* are prototrophic for leucine. 100 µL of 1:10,000 diluted culture was plated.

	Hours Of growth	3AT concentration	colonies counted per 0.01 ul of culture			
The medium used to conduct competitive growth.			SDWILV	SDW	Prototrophic for Leucine	Auxotrophic for Leucine
SGalWILV	0	0 mM	500	0	0	100
SGalWILV	24	0 mM 0.5 mM	288 576	0 0	0 0	100 100
SGalWILV	48	0 mM 0.5 mM	273 142	0 0	0 0	100 100
SGalWILV	72	0 mM 0.5 mM	1308 700	0 129	0 18	100 82
SGalWILV	96	0 mM 0.5 mM	1200 347	0 215	0 62	100 38
SGalWILV	120	0 mM 0.5 mM	743 1200	0 1248	0 100	100 0
SGalWILV	144	0 mM 0.5 mM	395 1100	33 928	8 84	92 16
SGalWILV	168	0 mM 0.5 mM	918 700	65 300	7 43	93 57

Unexpectedly, after 120 hours of competitive growth, the ratio shifted from low abundance of strains expressing GST-RWDBD^{R2259A}-TRP1-myc to the strain being 100% abundant (see Figure 3.14).

To verify our finding, we selected 36 colonies from the SGluWILV plate for our competitive growth. The SGluWILV plates support the growth of yeast strains no matter whether they are leucine auxotrophic or not. The 36 colonies were named as 33.04.01 to 33.04.18 and 33.05.01 to 33.05.18, the numbers representing the experiment number (33), the day of the competitive growth (day 04 [96 hours of growth]) or day (05 [120 hours of growth]), and the colony number. All thirty-six strains were screened for their ability to activate Gcn2. For this, the colonies were subjected to semi-quantitative growth assays. In this assay, yeast strains expressing GST-RWDBD-TRP1-myc $^{leu2\Delta}$, GST-RWDBD^{R2259A}-TRP1-myc, and GST alone, were used as a control, as well as yeast strain with the Gcn1 deletion (see Figure 3.15).

On observation, we showed that all 36 strains were able to overcome starvation and were prototrophic for the amino acid tryptophan and leucine (see Figure 3.15, see appendix 5.2 SGluW plate, SDWILV plate). This suggested that all strains expressed GST-RWDBD^{R2259A}-TRP1-myc. (see Figure 3.15, SGluW and SGalILV, see appendix 5.2). We also confirmed that the observed expression of the GST-RWDBD-TRP1-myc $^{leu2\Delta}$ and GST-RWDBD^{R2259A}-TRP1-myc did not itself result in a growth defect, because there was no impaired growth detectable on plates containing galactose but not 3AT (see Figure 3.15, SD plate). The Gcn1 deletion strain (*gcn*1Δ) was used to confirm that starvation was induced via the drug 3AT. This deletion strain was unable to overcome starvation. This confirms that the used 3AT concentrations were able to induce starvation for amino acid histidine in yeast (see Figure 3.15).

For further verification of our findings, we conducted a PCR based test. The approach was to amplify a portion of the RWDBDs containing the area of R2259, directly from the yeast competitive growth culture (cell culture PCR) followed by a restriction digest. This digest was used to determine the ratio of yeast strains expressing mutated RWDBD (performed as mentioned in section 2.11a and will be discussed in detail in 3.4 section of result) versus the wildtype RWDBD. The wildtype RWDBD domain contains the *AseI* restriction site near to the R2259 region of the RWDBD amplicon, but the RWDBD-R2259A amplicon does not.

For the control group (0 mM 3AT), when aliquots from the competitive growth cell culture were amplified through cell culture PCR, we observed a single band at 530 bp, as expected (see Figure 3.16). These amplicons were digested via *AseI* and resolved on 1.2% agarose gel.

After resolving, we showed that at 0-hour, three bands were seen on the gel at 530 bp, 365 bp and 125 bp (see Figure 3.17A). This suggested that our starter culture contained both wildtype and mutated RWDBD domain expressing strains. Similar results were observed for all the aliquots which were taken after every 22-24 hours of incubation till 144 hours of competitive growth (see Figure 3.17A Lanes 9, 12, 14,17,18, 20 and 23). This suggested that when competitive growth was carried out in the absence of 3AT, most of the strains could express wildtype RWDBD but not mutated RWDBD. As a result, yeast strains expressing mutated RWDBD were not enriched in the absence of 3AT.

For competitive growth on 0.5 3AT, at 0 hours, after PCR and RE digest, three bands were seen on the gel at 530 bp, 365 bp and 125 bp. This indicated that our starter culture contained both wildtype and mutated RWDBD domain expressing strains. Similar results were observed until 48 hours of competitive growth, as expected (see Figure 3.17A, lane 15).

At 72 hours, when the PCR amplicon was digested by *Ase*I and resolved on 1.2% agarose gel, we observed a predominant single band at 530 bp on the gel (see Figure 3.17A, lane 17). This suggested that most of yeast strains were expressing mutated RWDBD. Similar results were observed for aliquots taken after 72 hours until 144 hours of competitive growth (see Figure 3.17A, lanes 19, 21 and 22). Moreover, in contrast to the 0 hours competition, after 72 hours of competition only the 530 bp band was visible suggesting that most strains were expressing mutated RWDBD. This indicates that the yeast strains expressing mutated RWDBD were enriched in the presence of 3AT.

For more in-depth analysis, we calculated the intensity of the 530 bp and 125 bp bands of all samples using ImageJ software, and the probable ratio of strains expressing mutated RWDBD domain was determined. To consider that the 125 bp DNA fragment is 4.24 times smaller than the 530 bp DNA fragment observed on the gel, the signal intensity of the 125 bp band was multiplied by 4.24. The 530 bp band represents the mutated RWDBD domain whereas the band observed at 125 bp denotes wildtype RWDBD on digestion. A graph was plotted (see Figure 3.18). The graph suggested that at the end of 48 hours of competition on 0.5 mM 3AT culture, most yeast strains were expressing the mutated RWDBD domain (see Figure 3.18). In contrast to that, the leucine prototrophy test showed that most of yeast strains were expressing wildtype RWDBD (see Figure 3.14A). This will be discussed in more detail in Chapter 4.

To test whether the above findings were reproducible, the competitive growth was repeated. When the competitive growth was repeated, we plated a higher number of colonies for our

leucine prototrophy test to detect low abundant *LEU2+* strains. In fact, we could detect *LEU2*$^{+}$ strains on the SGluW plate from 24 hours of competitive growth (see Figure 3.19). This suggested that the observed discrepancy in the previous assay could be due to plating a low number on colonies to detect the representative.

To summarise, conducting the competitive growth assay for 120 hours with 0.5 mM 3AT will allow the enrichment of yeast strains expressing the mutated RWDBD domains which have lost ability to bind to Gcn2.

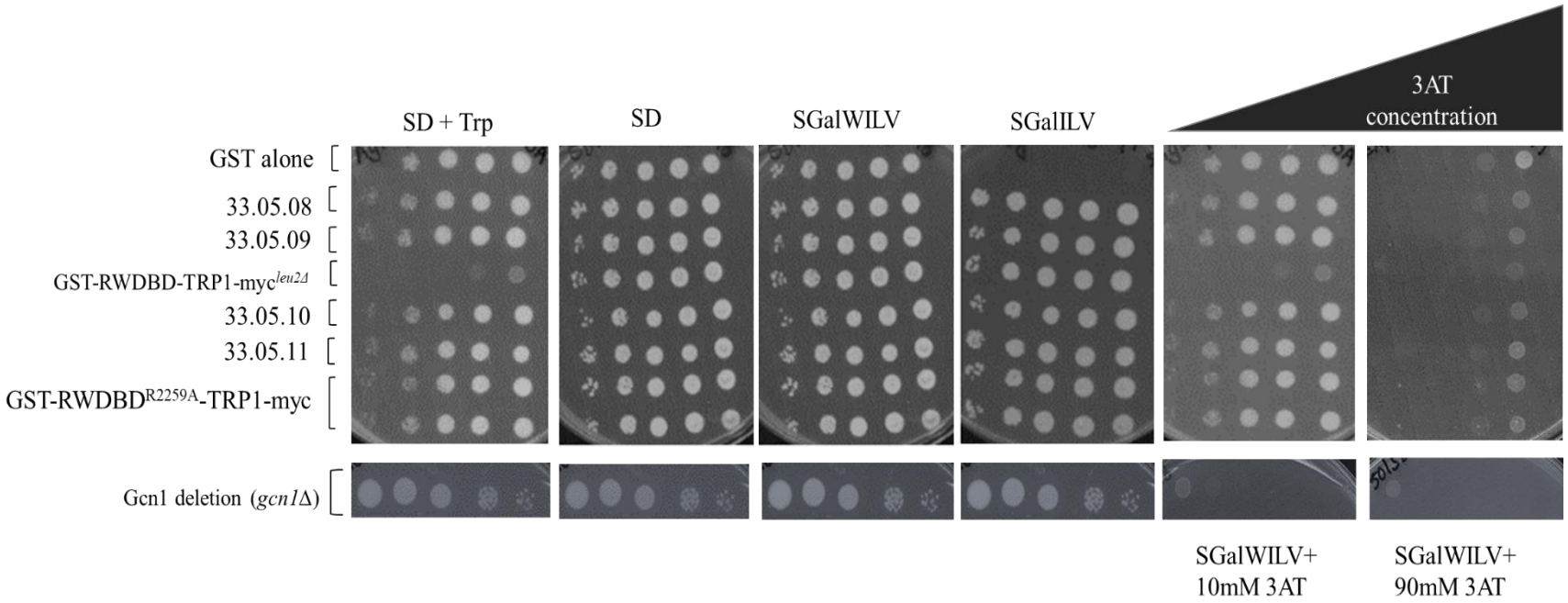


Figure 3. 15 Enriched strains expressed mutated RWDBDs and can overcome the starvation.

5 µL of ten-fold serially diluted overnight grown cultures of yeast cells isolated from the competitive growth culture (indicated by numbers 33.04.01) containing not-yet characterised plasmids, and yeast cells containing known plasmids as indicated, were transferred onto solid media containing 3AT. Incubation showed that the enriched colonies could overcome the starvation response (for other strains, see appendix Figure 5).

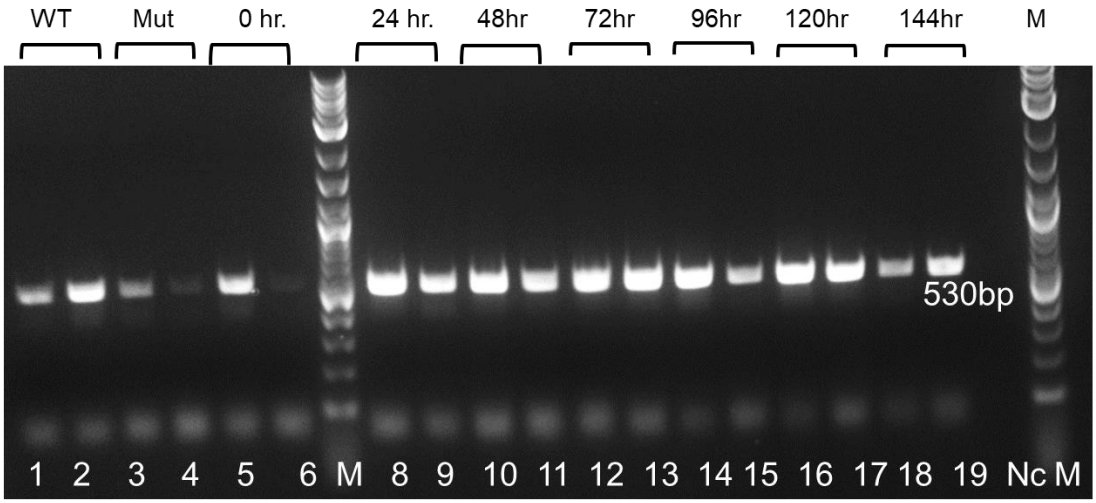


Figure 3. 16. Amplification of the RWDBD domain from cell culture.

The RWDBD domain was amplified out from the cell culture of competition assay after every 24 hours of incubation through the colony PCR reaction. The primers used for the amplification are ES2964 and ES2963. After completion of colony PCR, the product was visualized via gel electrophoresis on a 1.5 % agarose, and the amplified band was observed at 530 bp. Appropriate controls were used. (lane 1 wild type RWDBD domain and 2 mutated RWDBD domain)

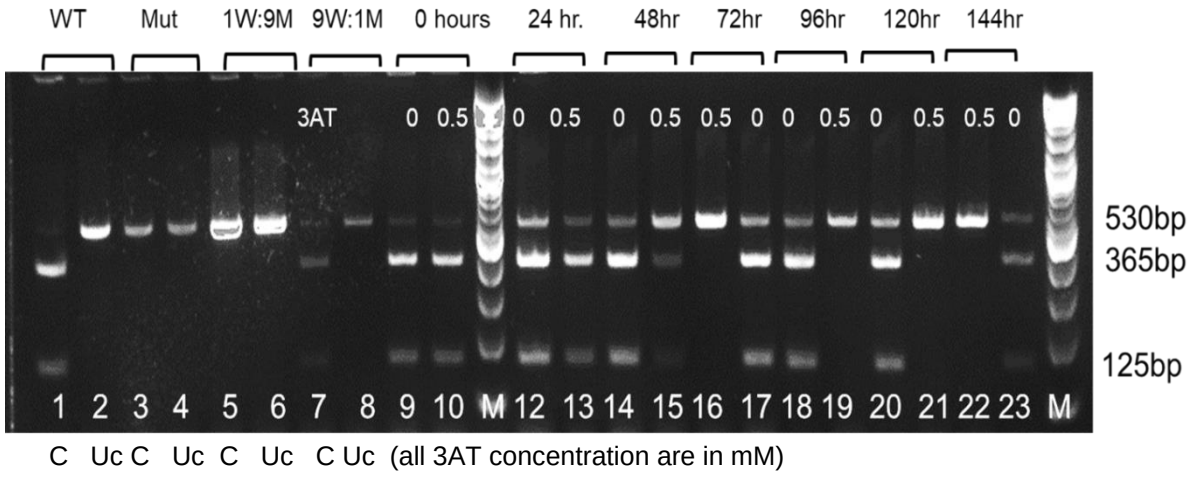


Figure 3. 17A An amplified RWDBD domain from competition assay was digested using AseI enzyme.

Two different bands were observed at 365 and 165 bp for 0 mM 3AT concentration till 144 hours of competition assay, whereas, only one single band was observed at 530 bp from 72 hours of competition assay for 0.5 mM 3AT, suggesting presences of mutated RWDBD domain (3AT ^{R}). Lane 1: digested wildtype RWDBD domain, Lane :2 Undigested wildtype RWDBD, Lane 3: digested GST-RWDBD ^{R2259A}-TRP1-myc domain, Lane 4: Undigested GST-RWDBD^{R2259A}-TRP1-myc domain, Lanes 5 and 6 indicate the artificial shift 1:9 ratio of 3AT ^{S} to 3AT $^{R.}$

Lanes 7 and 8 indicate the initial ratio of culture used as inoculant at 0 hours of competition assay. A shift in the ratio was observed at lane 16, which is after 72 hours of incubation.

Key: C ➔cut /digested by the enzyme Uc ➔ Undigested plasmid

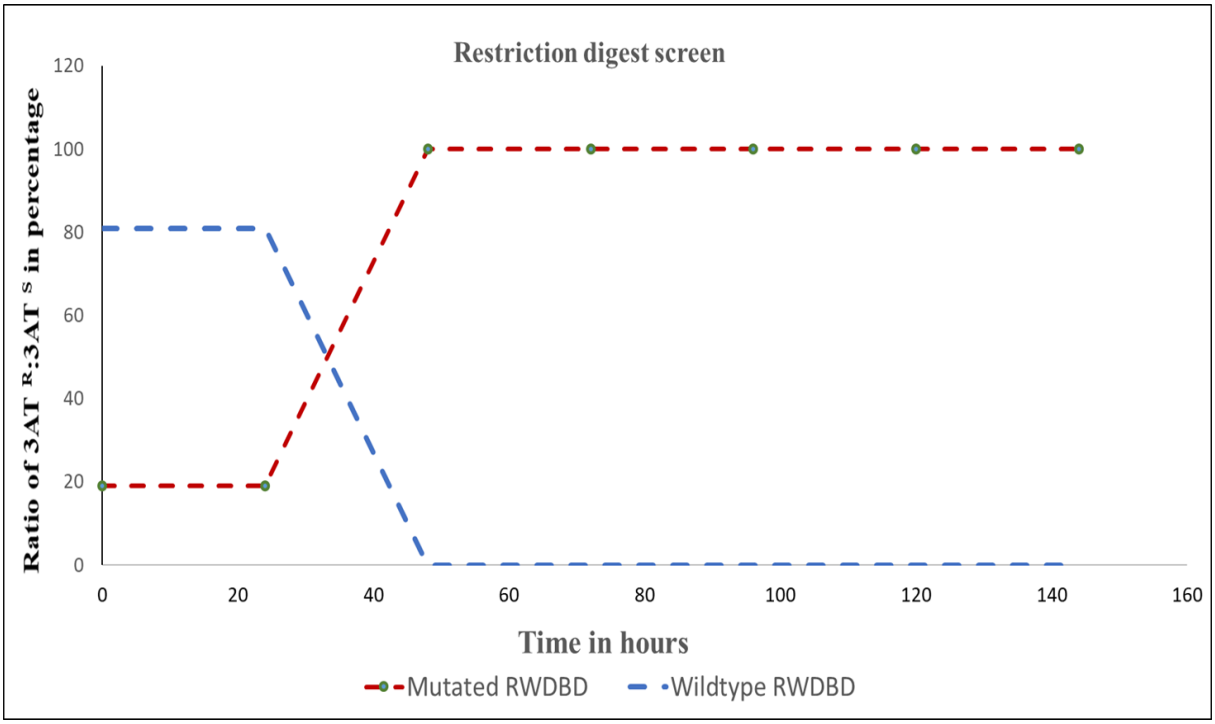


Figure 3. 18. The ratio of mutated RWDBD to wildtype RWDBD.

According to above graph, at 48 hours most of the strains are expressing mutated RWDBD domain. The calculated values are probable ratio. Note. The band observed for 0 mM 3 AT for 48 hours is very faint and out of linear range and is difficult to calculate the intensity through ImageJ software.

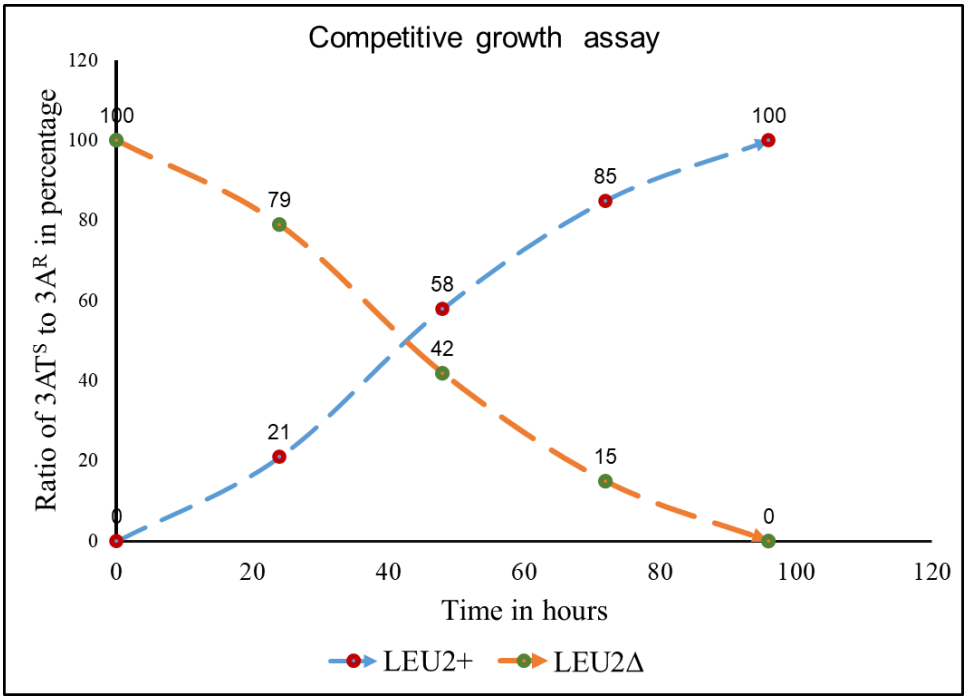


Figure 3. 19 The ratio of *LEU2*$^{+}$ *to LEU2*Δ during competitive growth.

As the incubation hours of competitive growth increased the yeast strain expressing *LEU2*Δ decreased, and the ratio of yeast strain expressing *LEU2*$^{+}$ increased.

3.4 Optimising the retrieval of sequence coding for mutated RWDBDs which have lost the ability to bind to the Gcn2 binding region

In the section above, we showed that competitive growth allows a faster selection and enrichment of mutated RWDBDs which have lost the ability to bind to the Gcn2 binding region, via phenotypic advantage. Now it is necessary to gain knowledge of which amino acid sequences were mutated. One possible method could be to use the cell culture PCR reaction, which allows the amplification of mutated RWDBDs directly from the cell culture, without cell lysis. This PCR reaction from now on will be referred to as the cell culture PCR reaction.

For the optimization we used a mixture of two cell cultures which contain yeast cells expressing different RWDBD constructs, one containing a restriction site. PCR should amplify DNA from both constructs, and restriction digest of the amplicon will help to determine the proportion of amplicons amplified from one template versus the other. The designed primers, ES2964 and ES2963, flank the Gcn2 binding region in which the unique restriction site is present. The PCR was conducted on a freshly grown cell culture of strains expressing either GST-RWDBD^{R2259A}-TRP1-myc or GST-RWDBD-TRP1-myc $^{leu2\Delta}$. The PCR amplicon was resolved in a 1.5% agarose gel (prepared as mentioned in section 2.15 materials and method). A predominant single band was found at 530 bp for lanes 1,2,4,5,7 and 8 corresponding to the expected 530 bp size of the DNA fragment, suggesting that the PCR amplification was successful (see Figure 3.20). In lanes 3 and 6 non-template controls were loaded (Figure 3.20). Furthermore, the absence of a band seen for lane 6 confirms that the amplified product is correct and denotes the negative control of the reaction.

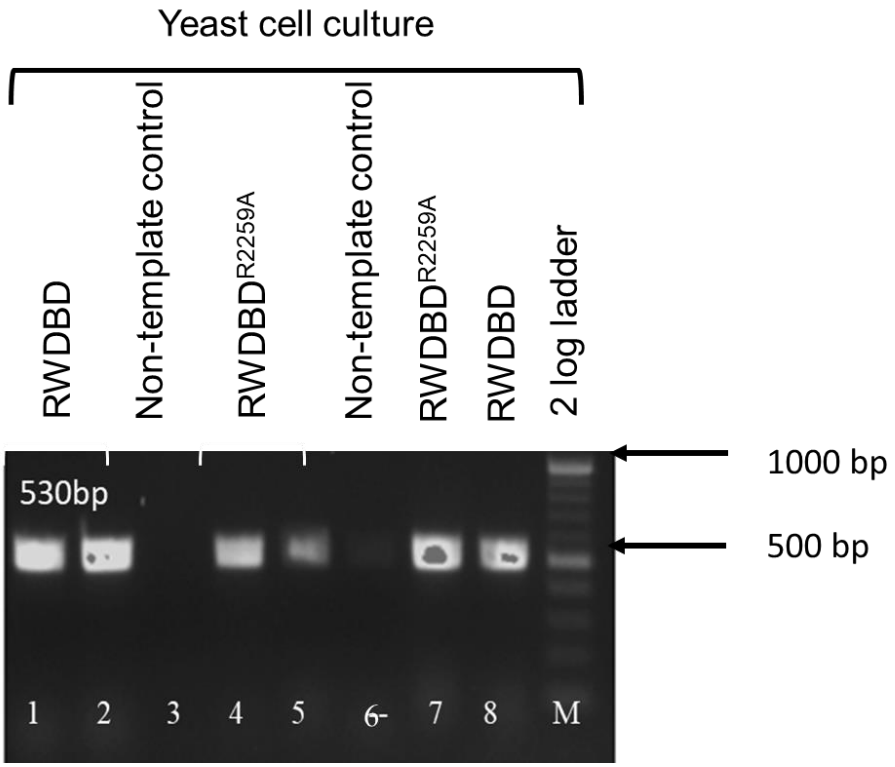


Figure 3. 20 Optimisation of cell culture PCR.

The RWDBD domain of Gcn1 was amplified using fresh cell culture grown in liquid medium. Primers ES2963 and ES2964 were used for amplification. An amplified band was observed at 530 bp on a 1.5% agarose gel.

On amplification, the single band at 530 bp does not help to differentiate between yeast strains expressing GST-RWDBD^{R2259A}-TRP1-myc and GST-RWDBD-TRP- myc $^{leu2\Delta}$. In our test, the GST-RWDBD-TRP- myc $^{leu2\Delta}$ contains a restriction site which is absent in the mutated one (see Figure 3.21). In this study, *AseI* (GCGAT^CGC) restriction enzyme was used, which cuts the RWDBD amplicon at the specific sequence (GCGAT^CGC). Thus, two DNA fragments (365 and 125 bp) were observed for the wildtype RWDBD amplicon after restriction digest (see Figure 3.22), whereas when yeast strains expressing GST-RWDBD^{R2259A}-TRP1-myc, were amplified and digested, they showed the presence of the mutation R2259A, results in loss of restriction sites. Therefore, a single predominant band was observed as expected (see Figure 3.22). Whereas, for the uncut amplicon, a single band was observed. This suggests that the *AseI*

restriction site was lost when the mutation was present in RWDBD of the yeast strain expressing GST-RWDBD ^{R2259A}-TRP1-myc (see Figure 3.22 and 3.21).

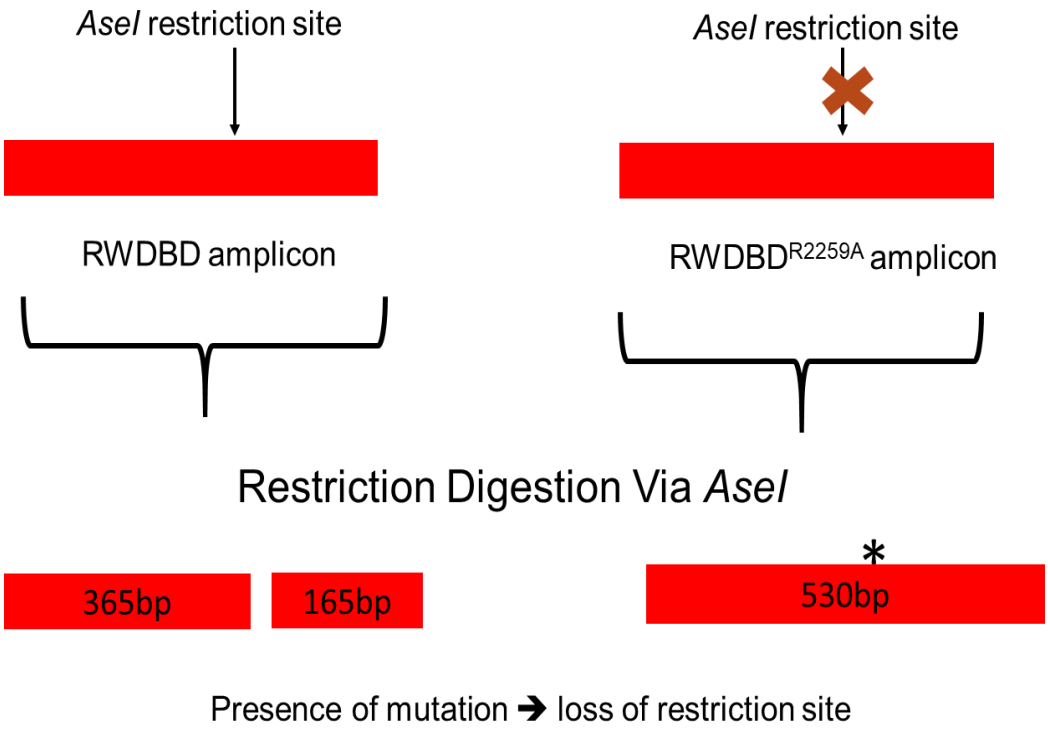


Figure 3. 21 The presence of the R2259A mutation is associated with the loss of restriction site for AseI.

The above diagram illustrates the presence and absence of R2259A mutation and the AseI restriction digest and amplification of a DNA fragment using primers ES2963, ES2964. When digested with AseI, two different DNA fragment will be obtained, depending on the presence of the restriction site, allowing the determination of whether the template DNA contained the R2259A substitution or not.

The restriction digest was optimised to determine the minimum amount of enzyme sufficient for the complete digestion of the PCR amplicon. For this, we used various amounts of enzymes (0.2, 0.3 and 0.4 µL) and performed the digestion as mentioned above. After the digestion we observed two bands at 365 and 125 bp on 1.5 % gel when 0.2 µL (2 units) of the enzyme was used. This suggests that 0.2 µL (2 units) was sufficient for complete digestion of the RWDBD amplicon (see Figure 3.20 B, lane 3).

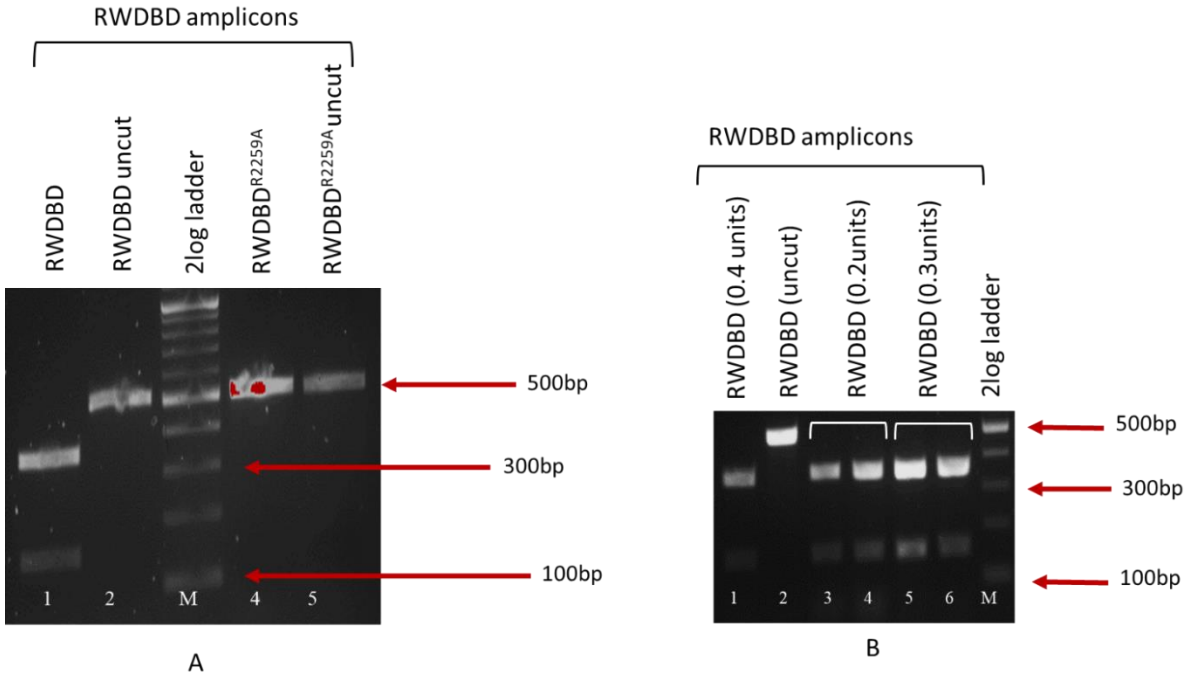


Figure 3. 22 Verification that the enzyme *AseI* can cut the RWDBD amplicon directly in the PCR reaction mix.

A An amplified RWDBD domain cleaved by *AseI* enzyme. Mutated RWDBD domain remains uncut. Non-mutated RWDBD domain was cleaved via the *AseI* enzyme, and two bands were observed at 365 and 165 bp on 1.5% agarose gel.

B A variable amount of enzyme was used to cleave the RWDBD domain. 0.2 µL of the enzyme (2 units) was sufficient to perform restriction digest. The uncut RWDBD domain was used as a negative control (lane 2). The digested bands were visualised via 1.5% agarose gel electrophoresis.

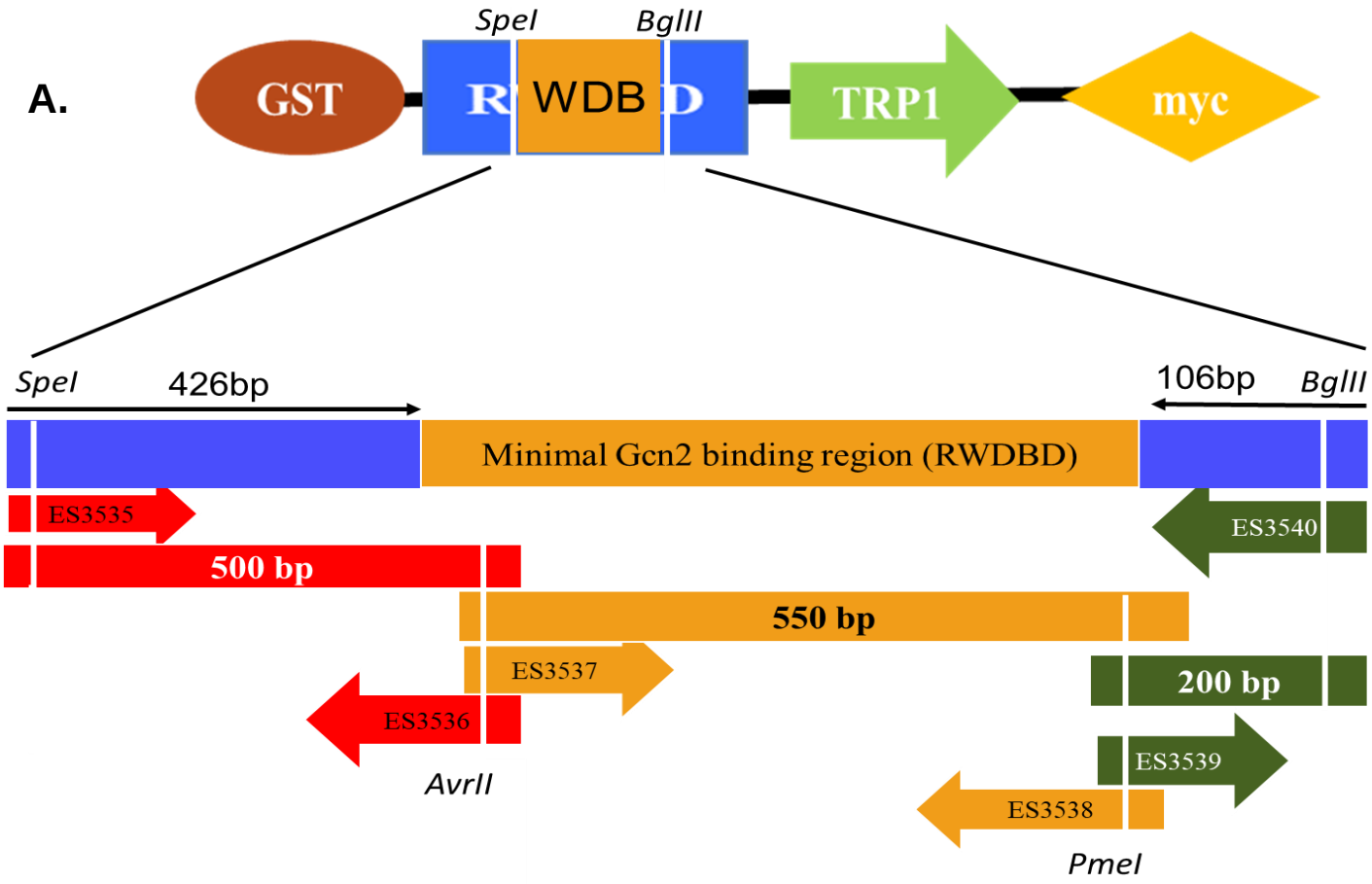
In addition to this, we also performed a PCR reaction to amplify DNA from a mixed culture (both GST-RWDBD-TRP- myc $^{leu2\Delta}$ and GST-RWDBD^{R2259A}-TRP1-myc expressing strains were mixed) and performed a restriction digest of the amplicon. For this PCR reaction, we have used a cell culture mixed in a 9:1 ratio (90% yeast strain expressing GST-RWDBD-TRP- myc $^{leu2\Delta}$:10% yeast strains expressing GST-RWDBD^{R2259A}-TRP1-myc) and 1:9 (10% yeast strains expressing GST-RWDBD-TRP-myc $^{leu2\Delta}$: 90% yeast strains expressing GST-RWDBD^{R2259A}-TRP1-myc). After amplification, a single band at 530 bp was seen, as expected. The amplicon of the 9:1 ratio (90% yeast strain expressing GST-RWDBD-TRP- myc $^{leu2\Delta}$:10 % GST-RWDBD^{R2259A}-TRP1-myc), was digested and showed three bands at 530 bp, 365 bp and 125 bp. This indicates that the RWDBDs were successfully amplified from the mixed culture. The 530 bp band represents GST-RWDBD^{R2259A}-TRP1-myc, bands at 365 and 125 bp denote yeast

strains expressing the GST-RWDBD-TRP- myc $^{leu2\Delta}$ construct (see Figure 3.17), whereas, for 1:9 (10% yeast strains expressing GST-RWDBD-TRP- myc $^{leu2\Delta}$: 90% yeast strains expressing GST-RWDBD^{R2259A}-TRP1-myc), we saw a single band at 530 bp. To summarise, we have optimised the process to retrieve coding sequences of the mutated RWDBD domain which can later be sent in for sequencing analysis.

3.5 Insertion of unique restriction sites that flank the Gcn2 binding region.

To easily replace the minimal Gcn2 binding region with a version that contains mutations which have lost the ability to bind to Gcn2, we need restriction sites that can immediately flank the minimal Gcn2 binding region.In the available plasmids pAG01, unique restriction sites were found that flank the Gcn2 binding region: *SpeI* and *BglII*. However, these available restriction sites were 426 bp and 106 bp away from the minimal Gcn2 binding region, respectively (see Figure 3.23). This meant that we required unique restriction sites which immediately flank the minimal Gcn2 binding region by incorporating *AvrII* and *PmeI*. We planned the incorporation of these restriction sites by introducing silent mutations using PCR (see Figure 3.23). To generate the DNA portion that has these immediately flanking restriction sites, three individual PCR reactions were performed.

The first PCR reaction, denoted as **A,** amplifies the DNA portion between the upstream existing unique restriction sites and the minimal Gcn2 binding region, using primers ES3535 and ES3536. The second PCR reaction, denoted as **B,** amplifies the minimal Gcn2 binding region using primers ES3537 and ES3538. Moreover, the region between the minimal Gcn2 binding region and downstream existing restriction site was amplified using primers ES3539 and ES3540, denoted as **C**. The primers ES3536 and ES3537 share a homologous region that contain the silent mutations which lead to the generation of the *AvrII* restriction site upstream of the minimal Gcn2 binding region (see Figure 3.23). Similarly, the primers ES3538 and ES3537 insert the *PmeI* restriction site, but downstream of the minimal Gcn2 binding site (see Figure 3.23).


A.
SpeI
BglII
GST
R
WDB
D
TRP1
myc
SpeI
426bp
106bp
BglII
Minimal Gcn2 binding region (RWDBD)
ES3535
ES3540
500 bp
550 bp
ES3537
200 bp
ES3536
ES3539
AvrII
ES3538
PmeI

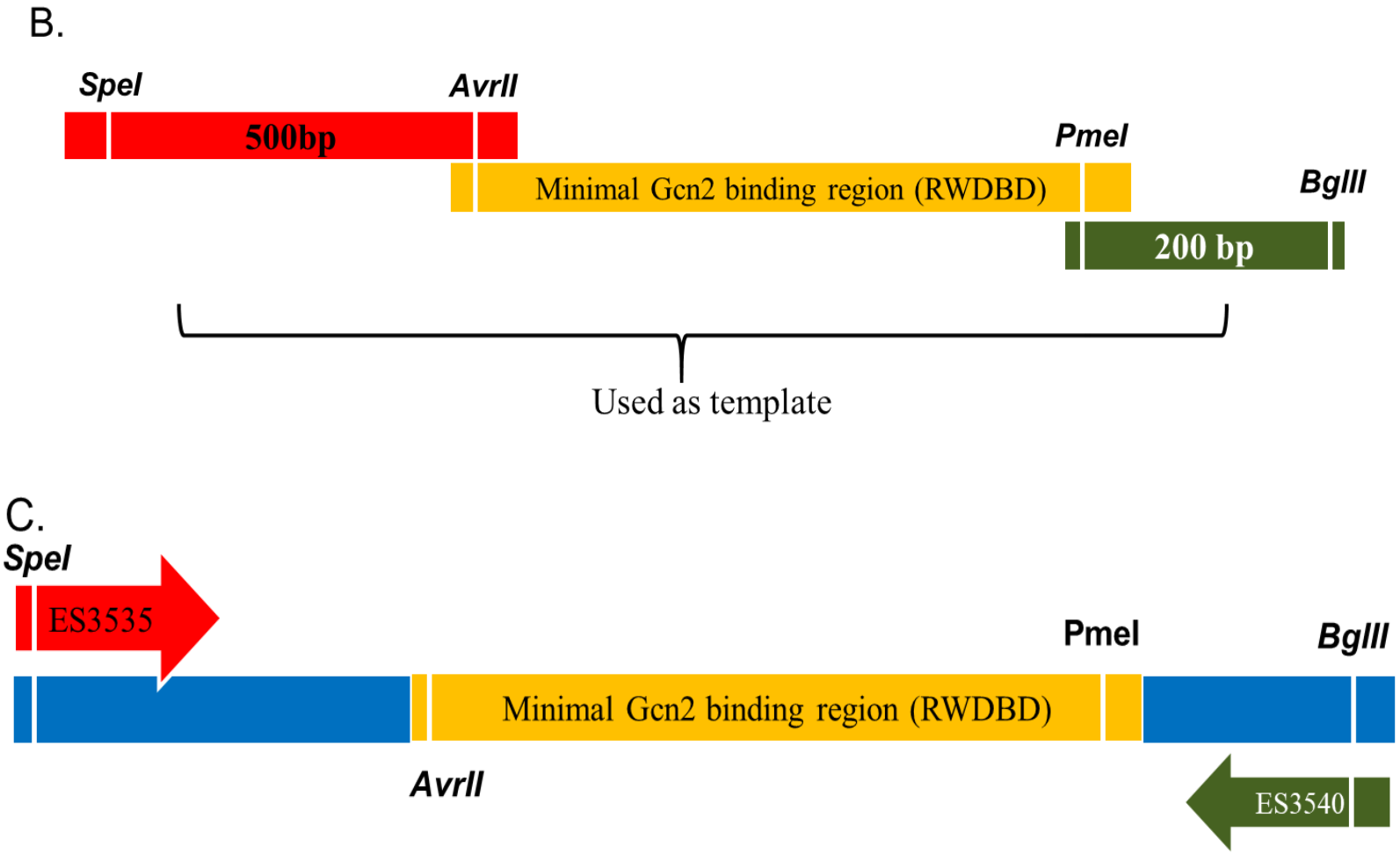

B.
SpeI
AvrII
500bp
PmeI
Minimal Gcn2 binding region (RWDBD)
BglII
200 bp
Used as template
C.
SpeI
ES3535
PmeI
BglII
Minimal Gcn2 binding region (RWDBD)
AvrII
ES3540

Figure 3. 23 Illustration for insertion of the AvrII and PmeI restriction site through fusion PCR reaction.

A. Three different PCR reactions were carried out. For insertion of *AvrII*, primers ES353 and ES3537 were used. For insertion of *PmeI*, primers ES3538 and ES3539 were used. **B.** The amplified product of all three reactions is then used as a template DNA for the fusion PCR reaction. **C.** Fusion PCR reaction, amplified using primers ES3535 and ES540.

The amplified products were resolved on 1.2 % agarose gel using electrophoresis. We observed DNA bands at the size of 500 (lanes 1 and 2), 550 (lanes 4 and 5) and 200 bp (lanes 8 and 9) (see Figure 3.24). These were the expected sizes for the DNA portion between the upstream existing unique restriction site and the minimal Gcn2 binding region, the portion between the minimal Gcn2 binding region and the downstream existing unique restriction, suggesting that we successfully amplified these DNA regions (see Figures 3.23 and 3.24). The faint band at 550 bp observed in lane 6 of Figure 3.24 could be the result of a spill over of the amplified product from lane 5 and lane 10. The band observed at lane 10 in Figure 3.24, may be due to primer-dimer. Primer-dimer could be observed due to a complementary base pair within primers.

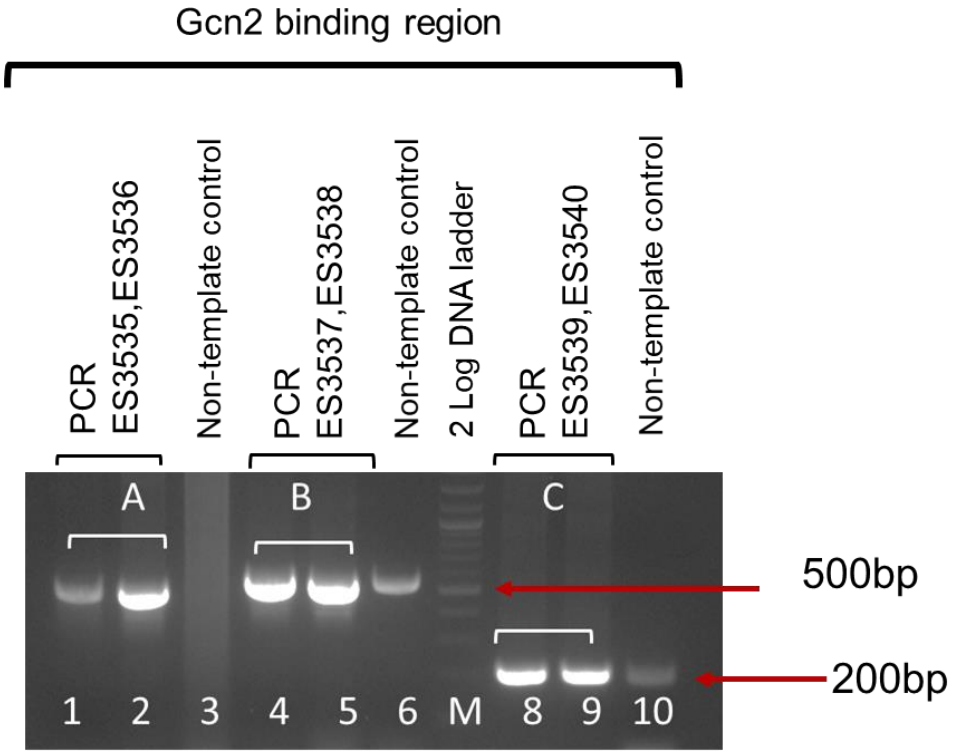


Figure 3. 24 Optimization of individual PCR reactions for insertion of restriction sites.

The sets of primers were used to insert AvrII and PmeI restriction sites through PCR amplification. The AvrII restriction site is inserted via amplifying the upstream Gcn2 binding regions (ES3545 and ES3536), minimal Gcn2 binding regions (ES3537 and ES3538) and PmeI inserted downstream of the Gcn2 binding region. (ES3539, ES3540) The amplified products were resolved on a 1.2% agarose gel, and three different bands were

observed at 500 bp, 550 bp and 200 bps. Lanes 3, 6 and 10 are negative controls. A 2log DNA ladder was used as a reference.

The next step was to conduct fusion PCR as outlined in the material method section 2.11b to join all three DNA fragments together using primers ES3535 and ES3540. The PCR reaction was resolved on 1.2% agarose gel through electrophoresis, and we observed a single band at 1200 bp, which is the expected size, suggesting the fusion PCR reaction was successful (see Figures 3.25 and 3.23, step 3). Now, this fused PCR product was used for cloning. For the preparation of the insert, the fusion PCR reaction was purified and digested with *BglII* and *SpeI* enzyme and resolved on 1.2% agarose gel. The digested fusion PCR product was then purified and used as an insert for the ligation reaction as outlined in the material and method section, 2.18.

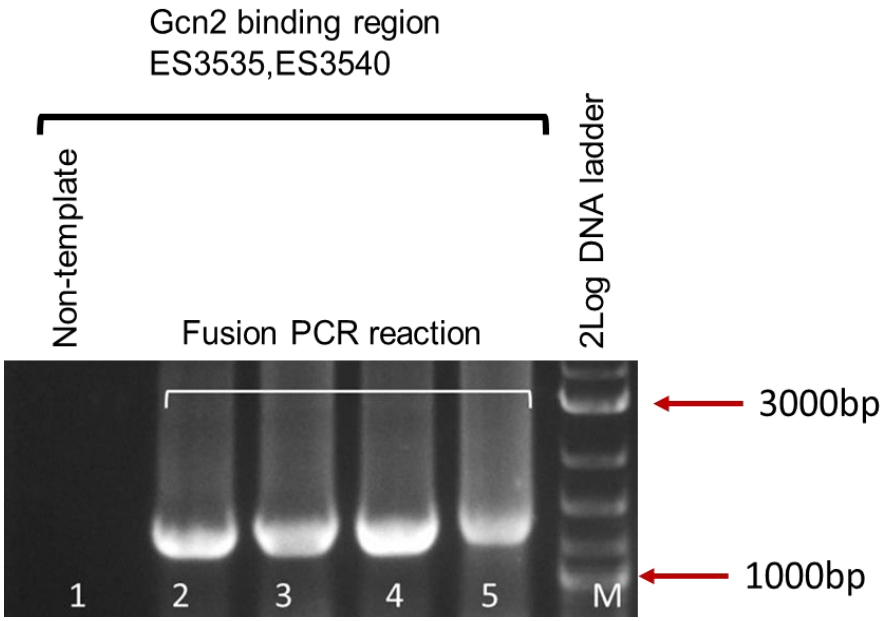


Figure 3. 25 Fusion PCR reaction with primers ES3535 and ES3540.

All three PCR products were used as template DNA and amplified using primers ES3535 and ES3540. An amplified product was resolved through gel electrophoresis. A predominant band was observed for samples at 1200 bp. The non-template was used as control. A 2log DNA ladder was used to denote band size. Lanes 2-5 are replicates of the fusion PCR reaction.

To replace the minimal Gcn2 binding region with the newly generated Gcn2 binding region, we must first cut open the pSG50 plasmid which contains the construct GST-RWDBD-TRP1-myc via *BglII and SpeI* restriction enzymes via restriction digest. The calf intestinal phosphatase (*CIP)* enzyme was added to the restriction reaction, to remove 5' phosphate from DNA and prevent self-ligation of the plasmid. To verify whether the digest was complete, an aliquot of the samples was resolved via electrophoresis on a 0.8% agarose. As a control the uncut plasmid was loaded along with digested plasmid on the gel (see Figure 3.26).

On resolving the samples, we observed a single predominant band at 9300 bp for *SpeI* and *BglII* digest enzymes which indicates cut plasmid. The bands seen at 10000 bp resemble the supercoil or relaxed circle states of the uncut plasmid. This confirms that the enzymatic action of *SpeI* and *BglII* has taken place in the double digest reaction. (see Figure 3.26). The digested plasmid was then purified as mentioned in section 2.13 of material and methods.

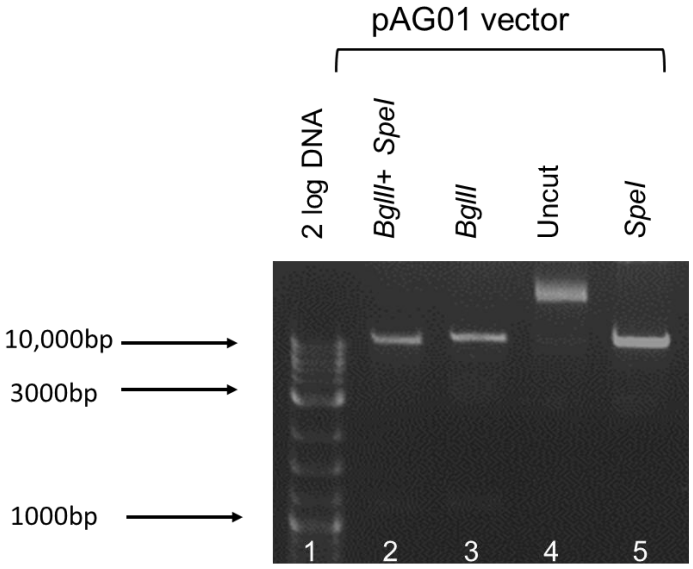


Figure 3. 26 Digestion of pAG01 with BglII, SpeI and CIP.

The plasmid was digested with the indicated restriction enzymes, and the samples were resolved using a 0.8 % agarose gel through electrophoresis. A 2log DNA ladder was used to denote band size.

The plasmid concentration was estimated by loading variable amounts (5 µL, 2.5 µL and 1 µL) on a 0.8 % agarose gel. 5 µL of 2 log DNA ladder (Biolabs, England) was loaded along with the plasmid samples. All loaded samples were resolved through electrophoresis. The gel was

then visualised, and the intensity of each DNA band was compared to the 2 log DNA marker. The amount of DNA was calculated as mentioned in section 2.16 of material and methods. For the ligation reaction, 3.5 µL (20 ng) of purified insert was calculated to have been used (See section 2.16 of material and methods for the calculation)

Both cut-open plasmid and insert were prepared for ligation reaction, as mentioned in section 2.18. After completion of the ligation reaction, the products were heated for 65℃, transformed into *E. coli* and plated on plates containing ampicillin (see section 2.7). The colonies showing ampicillin resistance were picked and were grown in LB with ampicillin liquid medium for 18 hours. The plasmid was extracted from the strains, as mentioned in section 2.16. The plasmids were verified for the presence of a restriction site through restriction digest. On digestion of plasmid, we observed a single predominant band for the sample digested by the *AvrII* and *PmeI* enzymes which indicates cut plasmid (see Figure 3.27). To ensure insertion of the new restriction sites did not introduce any mutations in the pAG02 plasmid, it needs to be sequence verified.

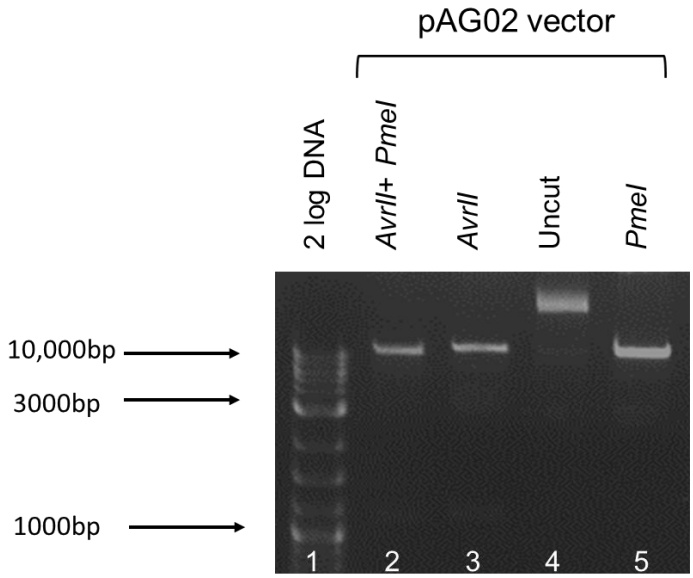


Figure 3. 27 Digestion of pAG02 with AvrII and PmeI restriction enzymes.

The plasmid was digested, and the bands were resolved using a 0.8 % agarose gel through electrophoresis. A predominant single band was observed in lane 2 indicating the presence of uncut plasmid, whereas the band in lane 1 indicates the presence of a double digested plasmid. The bands present in lanes 3 and 4 indicate successful digestion by each enzyme. A 2 log DNA ladder was used as reference to determine band size

3.6 Optimisation of random mutagenesis PCR

As we know, the random mutagenesis PCR inserts mutations through the error-prone PCR method non-specifically. We aim to optimise the mutagenesis procedure in a such a way that it shall have only 1-2 mutations in the Gcn2 binding region which is an RWDBD domain. In this section, parameters which are critical for mutation insertion will be optimised such as, easy incorporation of mutations in the minimal Gcn2 binding region as well as the number of mutations per 300 base pair of the Gcn2 binding region. Each section will be discussed in detail below.

3.6.1 Generation of mutated the Gcn2 binding region through fusion PCR reaction.

From previous studies, we know that R2259 is crucial for Gcn1-Gcn2 interaction. The modelled structure of the RWDBD suggests that the R2259 is surrounded by amino acids that are positively charged as R2259 is. This suggests that the Gcn1-Gcn2 interaction is ionic and is mediated by this positively charged amino acid patch within the Gcn1 RWDBD. This small stretch of 300 amino acids will be referred to as a minimal Gcn2 binding region. In this thesis, we aim to optimise conditions to mutagenize the minimal Gcn2 binding region to identify all possible amino acids involved in mediating the interaction (see Figure 3. 28).

To incorporate mutations in the minimal Gcn2 binding region, we used a genemorph II random mutagenesis kit. This kit consists of an enzyme called mutazyme II, a blend of DNA polymerase -mutazyme I and a novel *Taq* DNA polymerase. The novel *Taq* DNA has an advantage over the wildtype *Taq* DNA polymerase as it exhibits a high rate of mis-insertion and mis-extension frequency. This kit allows a mutational frequency of 1-16 mutations per kb with minimal mutational bias. With the genemorph II kit, low, medium, and high mutational frequencies can be achieved by altering the number of target DNA and amplification cycles. In this thesis, we aim to develop conditions that lead to the insertion of one to two mutations per 300 base pairs (see Table 3.2).

Table 3. 2 Random mutagenesis overview to insert mutation in minimal Gcn2 binding region. The data was deduced from the genemorph II manufacture's manual (Aligent™).

Base pairs (DNA)	Number of cycles	Number of mutations	Amount of target DNA (ng)
	High number	9-16 mutation/kb	low target DNA (0-500 ng)
1 KB	low number	4.5-9 mutations/ kb	high target DNA (500-1000 ng)
	High number	4-8 mutation/kb	low target DNA (0-250 ng)
500 bp	low number	2-3 mutations/ kb	high target DNA (250-500 ng)
	High number	2-4 mutation/kb	low target DNA (1-125 ng)
250 bp	low number	1-2 mutations/ kb	high target DNA (125-500 ng)

To achieve this mutational rate, we inferred the amount of template DNA and the number of amplification cycles required to insert mutations per 300 bp of the Gcn2 binding region (see table 3.2). For insertion of one to two mutations, we set our input template DNA to 125-500 ng with a low amplification number for 300 base pairs of the Gcn2 binding region.

For the generation of mutated Gcn2 binding regions through random mutagenesis PCR, we performed three different PCR reactions. For mutation insertion, we used primers ES3547 and ES3548, which amplifies the minimal Gcn2 binding region (see Figure 3.28). The amplified Gcn2 binding region is too short and it needs flanking sequences which can reach unique restriction sites. And immediately flanking the minimal Gcn2 binding region which furthermore allows the replacement of the wildtype sequence by the mutated ones (see Figure 3.28). Thus, primers ES3545, ES3546, ES3549, and ES3550 were used to amplify out the region upstream of Gcn2 binding and downstream of the Gcn2 binding region, respectively (see Figure 3.28).

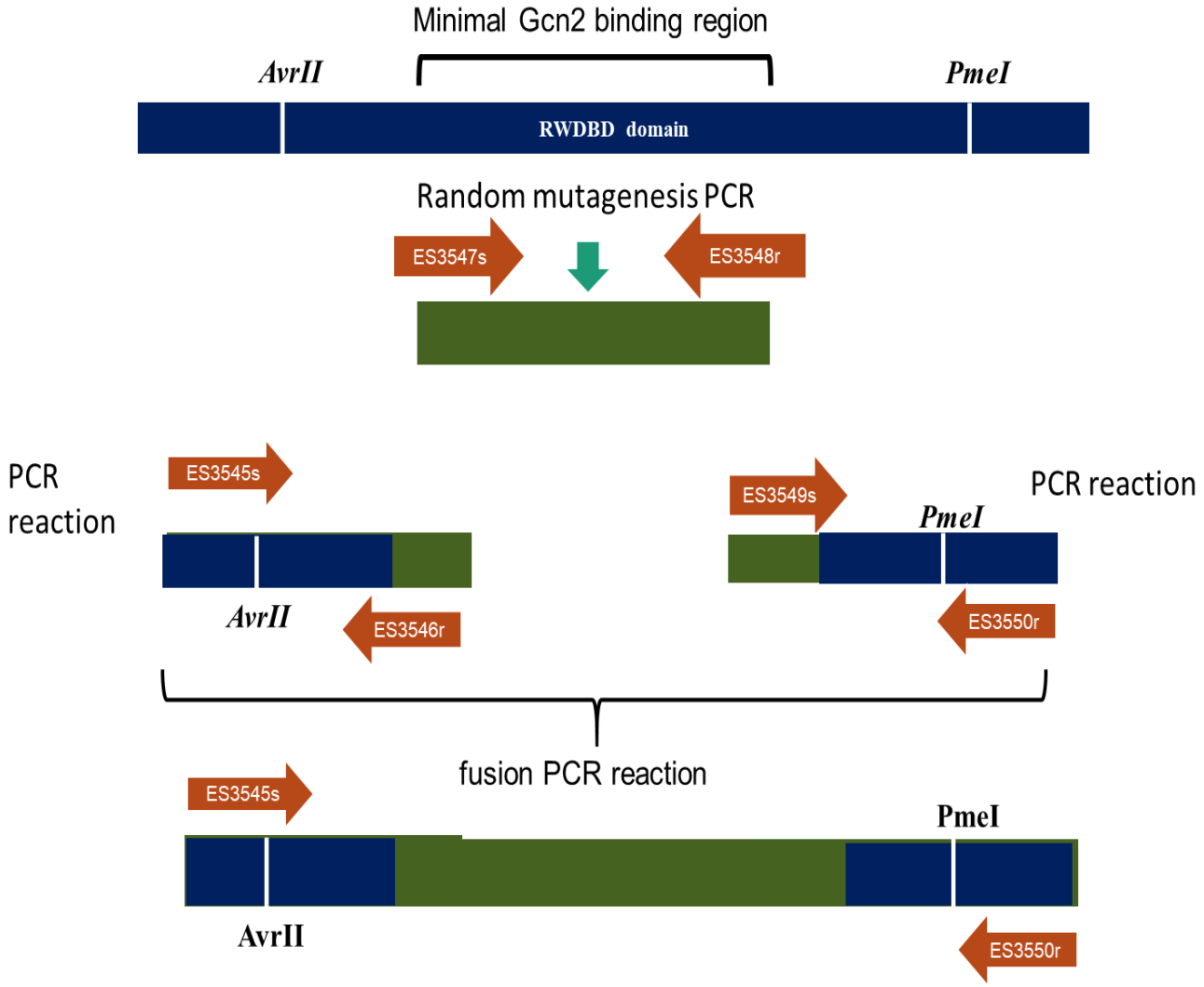


Figure 3. 28 : Generation of mutated Gcn2 binding regions through random mutagenesis PCR.

First, the minimal Gcn2 binding region will be amplified through primers ES3547 and ES3548, using random mutagenesis PCR. In parallel, the region upstream of the Gcn2 binding region and downstream of the Gcn2 binding region was amplified through primers ES3545, ES3546, ES3549, and ES3550, respectively. In the next step, all three PCR products were used as a template to generate full-length DNA via the fusion PCR reaction with primers ES3545 and ES3550.

The PCR reactions were carried out, and the amplicons were resolved on a 1.5% agarose gel through electrophoresis. We observed DNA bands at the size of 180, 300 and 300 bp in lanes 1,2,4,5,8 and 9, which had the expected size. The bands represent the upstream minimal Gcn2

binding region (180 bp) the minimal Gcn2 binding fragment (300 bp) and the downstream minimal Gcn2 binding fragment (300 bp), respectively, suggesting that we have successfully amplified these DNA regions (see Figure 3.29). The faint band at 180 bp observed in lane 3 of Figure 3.29 can be the result of a spill-over of the amplified product from lane 2. The very low bands observed in all lanes are likely to be primer-dimers (see Figure 3.29).

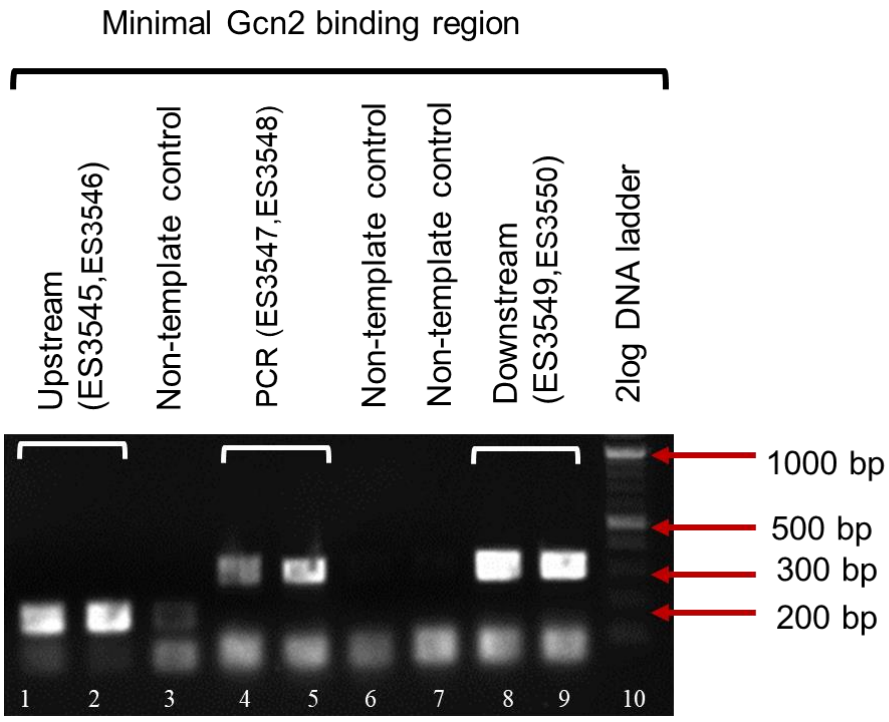


Figure 3. 29 Optimisation of individual PCR reactions.

Primer pairs were used to conduct individual PCR reactions as outlined in Figure 3.26. The amplified products resolved on lanes 3, 6 and 7 are negative controls. A 2 log DNA ladder was used as a reference.

A fusion PCR was performed using primers ES3545 and ES3550 to join all three PCR fragments together. The amplified products were resolved on 1.2% agarose gel through electrophoresis, and we observed no bands in the template and non-template lanes. This could be due to the high number of templates used in the PCR or the presence of excessive primers in the fusion PCR reaction. The excessive primers in the reaction could be from an aliquot of individual PCR reactions used as a template, which contained the primers from the previous PCR reaction. Therefore, in the next experiment, a purified PCR reaction was used as a template and the fusion PCR reaction was repeated.

To verify the PCR pieces were fused correctly during the fusion PCR reaction, the PCR products were loaded on 1.2% agarose gel and resolved via electrophoresis. We expected to see a PCR product of a size of 700 bp (see Figure 3.30). However, in addition to a 700 bp band, we observed two additional bands at 600 and 500 bp. (see Figure 3.30) These bands could be the result of two fragments joined instead of all three. The fusion of fragments 1 and 2 have an expected size of 500 bp, and fusion of fragments 2 and 3 have an expected size of 600 bp.

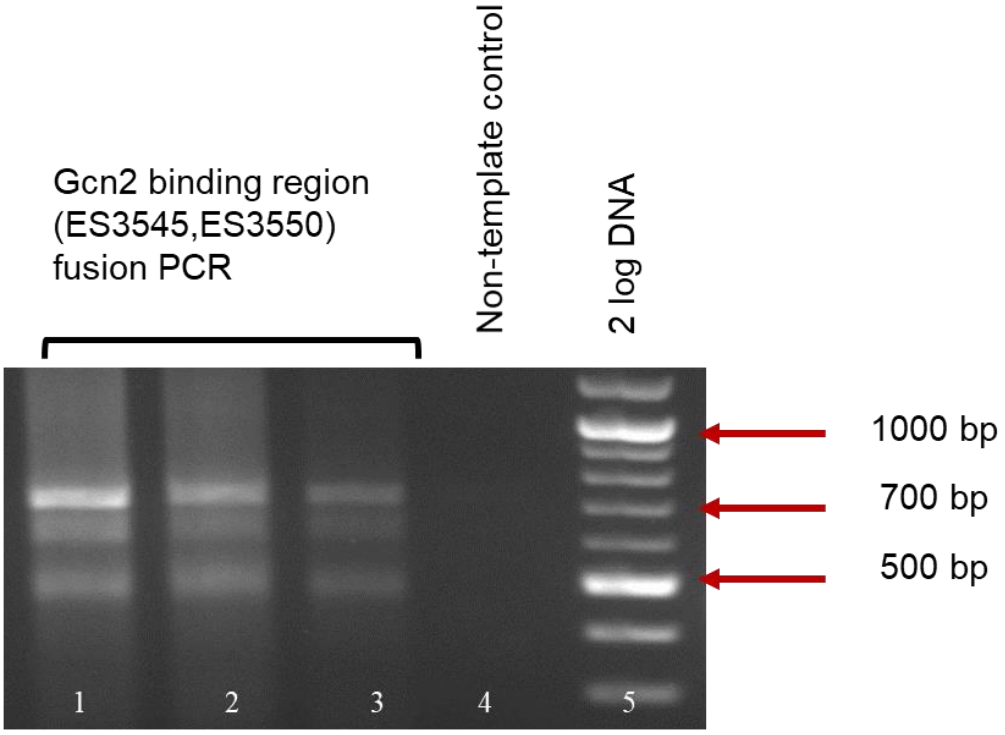


Figure 3. 30 Optimisation of fusion PCR reaction.

Primers ES3545 and ES3550 were used to generate a fusion PCR product, using the templates from Figure 3.27. The amplified products were resolved on 1.2% agarose. Lane 4 was the negative control lacking the templates.

Chapter 4 Discussion

4.1 Competitive growth for 120 hours is sufficient for enriching for yeast strains expressing mutated RWDBDs unable to bind Gcn2

The competitive growth was optimised using yeast strains expressing GST-RWDBD-TRP1-myc $^{LEU2\Delta}$ and GST-RWDBD^{R2259A}-TRP1-myc, respectively. Both strains were grown to saturation in SGluWILV medium for 48 h. To initiate the competitive growth, we have used a starter culture with 0.5 OD_{600} inoculated to SGalWILV which contained 0.5 mM 3AT. The starter culture contained nine parts of a yeast strain expressing GST-RWDBD-TRP1-myc $^{LEU2\Delta}$ and one part of yeast strain expressing GST-RWDBD^{R2259A}-TRP1-myc. In an experimental evolution assay the ratio likely is larger than 1:9, meaning that longer time selections likely will be required to enrich for strains with the desired phenotype. Here we chose a 1:9 ratio as an initial trial, however, before conducting a competitive growth experiment it may be desired to conduct growth time estimations using a ratio higher than 1:9.
An aliquot of the culture was taken after every 22-24 h of competitive growth and reinoculated into fresh liquid medium supplemented with 3AT (see Figure 3.13). Furthermore, to investigate the ratio of yeast strains expressing GST-RWDBD^{R2259A}-TRP1-myc, a tenfold serially diluted culture was plated onto SGluW and SGluWILV plates and incubated at 30℃ for 48 h (see Figure 3.13).

During the optimisation of the competition assay, we followed the abundance of strains expressing RWDBD^{R2259A} and wildtype RWDBD, identified by the strains ability of being *LEU2*$^{+}$ or *LEU2*Δ when grown on medium lacking leucine. As mentioned in Section 3.3.1, we had observed a discrepancy in abundance of LEU*2*$^{+}$ strains when comparing the data obtained from cell culture PCR and a restriction digest test and data from the leucine prototrophy screen test. One of the possible reasons for this could be the plating of low numbers of cells in the leucine prototrophy screen. When we repeated our competitive assay with identical growth conditions, but this time plating a higher number of cells to determine the number of leucine prototroph strains, we showed that this time we could detect the LEU2^{+} strains at the early hours of competitive growth (see Figure 3.18). This suggested that the observed discrepancy in the previous assay could be due to plating a low number on colonies to detect leucine prototroph strains.

To summarise, conducting the growth for 120 hours in presence of 0.5 mM 3AT is sufficient to enrich for yeast strains expressing mutated RWDBD that have lost the ability to bind to Gcn2.

4.2 Trp1 can be used as a selectable marker to eliminate truncated RWDBDs *in vivo*

As discussed in Section 3.1 of the results, we showed that *TRP1* could be used as a selectable marker. *TRP1* encodes for Phosphoribosyl anthranilate isomerase, which catalyses the third step in the tryptophan biosynthesis pathway and confers prototrophy for the amino acid tryptophan. In this study, we have fused the Trp1 to the C-terminal end of the RWDBD domain. We showed that a tryptophan auxotrophic strain expressing the RWDBD fused to Trp1 became tryptophan prototroph when growing on medium containing galactose, but not on medium containing glucose. This suggested that Trp1 is functional and only confers tryptophan prototrophy when the expression of the RWDBD is induced, in this case, induced by galactose (see Figure 3.4).

In summary, the placement of Trp1 after the RWDBD will help to eliminate any mutated RWDBD containing non-sense mutations. This is because the presence of non-sense mutations will lead to a truncated protein that lacks Trp1, meaning that the strain is no longer prototrophic for the amino acid tryptophan (see Figure 3.2).

4.3 An attempt to determine the cause of elevated growth of *LEU2* deletion strain after 120 h of competitive growth

As discussed in Section 3.3.1 of the Results, we had an unexpected finding during the competition assay optimisation. This was an increase in the number of yeast strains expressing the wildtype version of the RWDBD under starvation conditions, after longer than 120 h of competitive growth. This is surprising, because this strain should be unable to activate Gcn2, and therefore its growth should be slowed down under starvation conditions. The tryptophan prototrophy of these strains indicated that a full-length RWDBD was still expressed. Since these strains were leucine auxotrophic, this further indicated that the plasmid was the one containing the wildtype version of the RWDBD.

To confirm that these strains truly expressed the wildtype RWDBD, we have amplified the RWDBD domain using primer ES2964 and ES2963 from the cell culture for every 22 h of the assay. The amplified RWDBD amplicons were digested via *AseI* restriction enzyme. As mentioned in the 3.4 section of the result, the loss of *AseI* is observed due to the presence of mutations in RWDBD. On digestion these strains indeed did contain the wildtype RWDBD as two bands were observed on the gel at 365 and 125 bp which represent the wildtype RWDBDs (see Figure 3.17).

For elicited growth of a strain containing wildtype RWDBD that it can impair Gcn2 activation and now being able to grow under starvation conditions. One of the explanations would be that additional mutations have occurred in the yeast genome that allow the cells to again overcome starvation and grow well. For example, a mutation may have rendered Gcn2 constitutively active ($Gcn2^C$). It has been published that, for example, E1537 and E532K substitutions convert Gcn2 to $Gcn2^C$ (Manuel ramirez, 1992). Enhanced eIF2α phosphorylation associated with $Gcn2^C$ would allow an increased ability to respond to amino acid starvation, as seen by increased growth. Another possibility is the insertion of mutations (Arg794 and Phe842) in the protein kinase domain of Gcn2, which also converts Gcn2 to $Gcn2^C$ and even eliminates the need for tRNA binding for Gcn2 activation(Carlos r. Vazquez de aldana, 1994,). This allows Gcn2 to trigger an amino acid response without Gcn1-Gcn2 interaction. However, since the goal of this thesis was to establish a screening procedure, the cause of this observed phenomenon was not further investigated.

Despite this odd phenomenon, the colonies tested at 96 and 120 h of competition assay confirmed the increased presence of strains containing mutated RWDBD domains (see Figure 3.15 and appendix 5.3 to 5.6). This suggests that 120 h of competition assay is sufficient for the enrichment procedure. Therefore, the observed sudden growth phenomenon likely will have had no adverse effect on the screening process.

4.4 The established screening method is well suitable for determining amino acids in Gcn1 involved in Gcn2-interaction

The screening procedure established in this thesis allows the enrichment of yeast strains expressing mutated RWDBDs, because such strains experience gain of growth, while strains expressing RWDBDs able to bind Gcn2 have reduced growth. As mentioned in 1.12 section of introduction, when mutated RWDBDs are overexpressed in yeast cells, that lost its ability to bind to Gcn2, the yeast cells are again able to activate Gcn2 to overcome the starvation caused by 3AT.

Just looking at the strains' growth behaviour, we can determine whether the inserted mutations affect Gcn1-Gcn2 interaction or not.

Another advantage of our method is the possibility of accumulating mutations by conducting repetitive cycles of random mutagenesis PCR and competitive growth. Through accumulation of more mutations in the RWDBD, we can mutate in the same RWDBD all amino acids which are relevant for mediating Gcn1-Gcn2 interaction. Thus, allowing us to comprehensively identify the amino acids which are involved in Gcn1-Gcn2 interaction. This procedure may also lead to the accumulation of changes of amino acids not relevant for Gcn1-Gcn2 interaction, however, conducting the screening method in 10 independent parallel approaches will help identify those. This is because the 10 parallel screening approaches would have in common the accumulation of amino acid mutations that affect Gcn1-Gcn2 interaction, but not amino acid mutations that are irrelevant for Gcn1-Gcn2 interaction.

The established screening procedure to identify the amino acids involved in mediating the Gcn1-Gcn2 interaction can also be used for comprehensively identifying amino acids relevant for protein-protein interactions of any two proteins, if growth conditions can be found that allow for the enrichment for the desired mutations.

4.5 Conclusion

In this study, we have developed a screening procedure for finding amino acids in Gcn1 that are important for protein-protein interaction between Gcn1 and Gcn2. We have developed a selection system which eliminates truncated RWDBD domains, by having Trp1 fusion in frame at the C-terminal of RWDBD, thereby conferring Trp prototrophy.

For enrichment and selection of mutated RWDBD domains unable to bind Gcn2, we have optimised the competitive growth. We showed that a 3AT concentration of 0.5 mM is most efficient to conduct the competitive growth, as it hampers the growth of yeast strains unable to activate Gcn2 and promotes the growth of yeast strains able to activate Gcn2 in starvation.

For easy insertion of mutations, the Gcn2 binding region within the RWDBD domain was flanked with new restriction sites recognized by *AvrII* and *PmeI*. To retrieve the sequences of the mutated Gcn2 binding region easily and efficiently, a cell culture PCR procedure was optimised.

To introduce one or two mutations per 300 bp in the minimal Gcn2 binding region of RWDBD domain via PCR, we have predicted the necessary number of amplification cycles and the amount of DNA required.

Once random mutagenesis PCR is optimised, the screen can be used to comprehensively identify amino acids involved in Gcn1-Gcn2 interaction. The established procedure will allow us to determine and map the interaction hotspot for Gcn2 in Gcn1. This novel method established here could be applied to similar studies aimed to uncover amino acids involved in protein-protein interactions.

4.6 Future Perspective

In this research, several parameters of the screening process for finding amino acid in Gcn1 relevant for Gcn2 binding have been optimised. One more parameter that remains to be optimised is random mutagenesis PCR.

When this remaining parameter has been optimised, the screening process can be performed to comprehensively identify the amino acids in Gcn1 that are involved Gcn2 binding. Once the screening process has been completed, meaning that the resulting growth culture predominantly contains yeast strains expressing RWDBDs unable to bind Gcn2, the culture will be plated to select a series of single colonies. From these strains, the sequence of their RWDBD domains will be determined to reveal the amino acid mutations. If the mutated RWDBD domain contains several mutations, then a single substitution of amino acids will be generated to reveal which amino acid is responsible for Gcn2 binding. Further testing for the relevance of these amino acids could be via protein-protein interaction studies such as co-immunoprecipitation studies or Yeast Two hybrid assays.

When the mutated RWDBD domain is expressed in the yeast cells, it is expected that the interaction between Gcn1-Gcn2 is disrupted. As a result, Gcn2 cannot get activated and cannot phosphorylate its substrate eIF2α. Therefore, determining the cellular levels of eIF2α-P in a quantitative manner will reveal which amino acids are (most) critical for Gcn1-Gcn2 interaction. Towards the end, this screen will reveal all amino acids relevant for Gcn2 binding in Gcn1 and show the exact location of the Gcn2 binding site in Gcn1. Moreover, the approach established in this research can be also used to determine the amino acids of the RWD domain of Gcn2 relevant for Gcn1 binding.

Findings from this current research combined with the above-mentioned future work will help in furthering knowledge of how Gcn1-Gcn2 interact. This will help to reveal a complete understanding of the Gcn2 function response to amino acid starvation and its involvement in other biological processes.

As a matter of interest, further testing could be done to determine the cause or possible mutation that have elicited the growth of wildtype RWDBD expressing strains after 120 ho of competitive growth in 3 AT.

References

Baird, T. D., & Wek, R. C. (2012). Eukaryotic initiation factor 2 phosphorylation and translational control in metabolism. *Adv Nutr, 3*(3), 307-321. 10.3945/an.112.002113

Berlanga, J. J., Ventoso, I., Harding, H. P., Deng, J., Ron, D., Sonenberg, N., . . . de Haro, C. (2006). Antiviral effect of the mammalian translation initiation factor 2alpha kinase GCN2 against RNA viruses. *EMBO J, 25*(8), 1730-1740. 10.1038/sj.emboj.7601073

Carlos R.Vazquez de Aldana, M. J. M. a. A. G. H. (1995). GCN20, a novel ATP binding cassette protein, and GCN1 reside in a complex that mediates activation of the elF-2a kinase GCN2 in amino acid-starved cells. *The EMBO Journal, vol.14* (13), 3184-3199,.

Carroll, B., Korolchuk, V. I., & Sarkar, S. (2015). Amino acids and autophagy: cross-talk and co-operation to control cellular homeostasis. *Amino Acids, 47*(10), 2065-2088. 10.1007/s00726-014-1775-2

Castilho, B. A., Shanmugam, R., Silva, R. C., Ramesh, R., Himme, B. M., & Sattlegger, E. (2014). Keeping the eIF2 alpha kinase Gcn2 in check. *Biochim Biophys Acta, 1843*(9), 1948-1968. 10.1016/j.bbamcr.2014.04.006

Clemens, M. J. (1994.). Regulation of eukaryotic protein synthesis by protein kinases that phosphorylate initiation factor elF-2. *Molecular Biology Reports*, 201-210.

Costa-Mattioli, M., Gobert, D., Harding, H., Herdy, B., Azzi, M., Bruno, M., . . . Sonenberg, N. (2005). Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. *Nature, 436*(7054), 1166-1173. 10.1038/nature03897

Devi, L., & Ohno, M. (2013). Deletion of the eIF2alpha Kinase GCN2 fails to rescue the memory decline associated with Alzheimer's disease. *PLoS One, 8*(10), e77335. 10.1371/journal.pone.0077335

G.Hinnebusch1, E. S. a. A. (2000). Separate domains in GCN1 for binding protein kinase GCN2 and ribosomes are required for GCN2 activation in amino acid-starved cell. *The EMBO Journal, Vol. 19*(No.23), pp. 6622±6633,.

Grallert, B., & Boye, E. (2007). The Gcn2 kinase as a cell cycle regulator. *Cell Cycle, 6*(22), 2768-2772. 10.4161/cc.6.22.4933

Hinnebusch, A. G. (1994). The eIF-2alpha kinases regulators of protein synthesis in starvation and stress. *cell biology, 05* 417-426. 1043-4682/94/060417+10$8.00/0

Hinnebusch, A. G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol, 59*, 407-450. 10.1146/annurev.micro.59.031805.133833

Hongfang Qiu, J. D., Cuihua Hu, Christopher S.Francklyn1 and Alan G.Hinnebusch2. (2001). The tRNA binding moirty in gcn2 contains a dimerization domain that interacts with kinase domain and is required for tRNA binding and kinase activation. . *The EMBO Journal, 20* (6), 1425±1438

Inglis, A. J., Masson, G. R., Shao, S., Perisic, O., McLaughlin, S. H., Hegde, R. S., & Williams, R. L. (2019). Activation of GCN2 by the ribosomal P-stalk. *Proc Natl Acad Sci U S A, 116*(11), 4946-4954. 10.1073/pnas.1813352116

James C. Jiang, E. J., Marina V. Repnevskaya, and S. Michal Jazwinski. (2000). Activation of GCN2 kinase by ribosome stalling links translation elongation with translation initiatio.pdf. *he FASEB Journal* 10.1096/fj.00-0242fje

Kimball, S. R. ((1999)). Eukaryotic initiation factor eIF2. *The International Journal of Biochemistry & Cell Biology, 31*, 25±29.

Koromilas, A. E. (2015). Roles of the translation initiation factor eIF2alpha serine 51 phosphorylation in cancer formation and treatment. *Biochim Biophys Acta, 1849*(7), 871-880. 10.1016/j.bbagrm.2014.12.007

Kubota, H., Ota, K., Sakaki, Y., & Ito, T. (2001). Budding yeast GCN1 binds the GI domain to activate the eIF2alpha kinase GCN2. *J Biol Chem, 276*(20), 17591-17596. 10.1074/jbc.M011793200

Kubota, H., Sakaki, Y., & Ito, T. (2000). GI domain-mediated association of the eukaryotic initiation factor 2alpha kinase GCN2 with its activator GCN1 is required for general amino acid control in budding yeast. *J Biol Chem, 275*(27), 20243-20246. 10.1074/jbc.C000262200

Lageix, S., Zhang, J., Rothenburg, S., & Hinnebusch, A. G. (2015). Interaction between the tRNA-binding and C-terminal domains of Yeast Gcn2 regulates kinase activity in vivo. *PLoS Genet, 11*(2), e1004991. 10.1371/journal.pgen.1004991

Lee, S. J., Swanson, M. J., & Sattlegger, E. (2015). Gcn1 contacts the small ribosomal protein Rps10, which is required for full activation of the protein kinase Gcn2. *Biochemical Journal, 466*(3), 547-559. 10.1042/BJ20140782

Liu, H., Huang, L., Bradley, J., Liu, K., Bardhan, K., Ron, D., . . . McGaha, T. L. (2014). GCN2-dependent metabolic stress is essential for endotoxemic cytokine induction and pathology. *Mol Cell Biol, 34*(3), 428-438. 10.1128/MCB.00946-13

Matthew j. Marton, d. c., t and alan g. Hinnebusch (1993). GCN1, a Translational Activator of GCN4 in Saccharomyces cerevisiae, Is Required for Phosphorylation of Eukaryotic Translation Initiation Factor 2 by Protein Kinase GCN2. *Molecular and cellular biology,, 13*(06), 3541-3556 0270-7306/93/063541-16$02.00/0

Manuel ramirez, t. r. c. W., 4 carlos r. Vazquez de aldana, belinda m. Jackson, barbara freeman,§ and alan g. Hinnebusch*. (1992). Mutations Activating the Yeast eIF-2Kinase GCN2 Isolation of Alleles Altering the Domain Related to Histidyl-tRNA Synthetases. *Molecular and cellular biology,, 12*(12), 5801-5815. 0270-7306/92/125801-15$02.00/0

Marton, M., Crouch, D., & Hinnebusch, A. (1993). GCN1, a translational activator of GCN4 in Saccharomyces cerevisiae, is required for phosphorylation of eukaryotic translation initiation factor 2 by protein kinase GCN2. *Molecular and Cellular Biology, 13*(6), 3541-3556. 10.1128/MCB.13.6.3541

Marton, M. J., Vazquez de Aldana, C. R., Qiu, H., Chakraburtty, K., & Hinnebusch, A. G. (1997). Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2alpha kinase GCN2. *Molecular and Cellular Biology, 17*(8), 4474-4489. 10.1128/MCB.17.8.4474

Matthew j. Marton, c. r. V. d. a., 1 kalpana chakraburtty,2 and alan g. Hinnebusch. (1997). Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2alpha kinase gcn2. *Molecular and cellular biology,, Vol. 17,* (No. 8), 4474–4489. 0270-7306/97/$04.00⩽0

Matthew j. Marton, c. r. V. d. a., 1 kalpana chakraburtty,2 and alan g. Hinnebusch. (Aug. 1997). Evidence that GCN1 and GCN20, Translational Regulators of GCN4, Function on Elongating Ribosomes in Activation of eIF2⎵ Kinase GCN2. *Molecular and cellular biology, , Vol. 17,* (No. 8), 4474–4489. 0270-7306/97/$04.00⩽0

Minerva Garcia-Barrio, J. D., Sandra Ufano1 and Alan G.Hinnebusch2. (, 2000). Association of GCN1-GCN20 regulatory complex with N-terminus of eIF2alpha kinase GCN2 is required for GCN2 activation. . *The EMBO Journal, Vol. 19*(No.8), pp. 1887±1899.

Padyana, A. K., Qiu, H., Roll-Mecak, A., Hinnebusch, A. G., & Burley, S. K. (2005). Structural basis for autoinhibition and mutational activation of eukaryotic initiation

factor 2alpha protein kinase GCN2. *J Biol Chem, 280*(32), 29289-29299. 10.1074/jbc.M504096200

Qiu, H., Hu, C., Dong, J., & Hinnebusch, A. G. (2002). Mutations that bypass tRNA binding activate the intrinsically defective kinase domain in GCN2. *Genes & Development, 16*(10), 1271-1280. 10.1101/gad.979402

Rakesh, R., Krishnan, R., Sattlegger, E., & Srinivasan, N. (2017). Recognition of a structural domain (RWDBD) in Gcn1 proteins that interacts with the RWD domain containing proteins. *Biol Direct, 12*(1), 12. 10.1186/s13062-017-0184-3

Ramirez, M., Wek, R. C., & Hinnebusch, A. G. (1991). Ribosome association of GCN2 protein kinase, a translational activator of the GCN4 gene of Saccharomyces cerevisiae. *Molecular and Cellular Biology, 11*(6), 3027-3036. 10.1128/mcb.11.6.3027

Ravishankar, B., Liu, H., Shinde, R., Chaudhary, K., Xiao, W., Bradley, J., . . . McGaha, T. L. (2015). The amino acid sensor GCN2 inhibits inflammatory responses to apoptotic cells promoting tolerance and suppressing systemic autoimmunity. *Proc Natl Acad Sci U S A, 112*(34), 10774-10779. 10.1073/pnas.1504276112

Roffe, M., Hajj, G. N., Azevedo, H. F., Alves, V. S., & Castilho, B. A. (2013). IMPACT is a developmentally regulated protein in neurons that opposes the eukaryotic initiation factor 2alpha kinase GCN2 in the modulation of neurite outgrowth. *J Biol Chem, 288*(15), 10860-10869. 10.1074/jbc.M113.461970

Sattlegger, E., Barbosa, J. A., Moraes, M. C., Martins, R. M., Hinnebusch, A. G., & Castilho, B. A. (2011). Gcn1 and actin binding to Yih1: implications for activation of the eIF2 kinase GCN2. *J Biol Chem, 286*(12), 10341-10355. 10.1074/jbc.M110.171587

Sattlegger, E., & Hinnebusch, A. G. (2000). Separate domains in GCN1 for binding protein kinase GCN2 and ribosomes are required for GCN2 activation in amino acid-starved cells. *The EMBO Journal, 19*(23), 6622-6633. 10.1093/emboj/19.23.6622

sheree a. wek, s. z., and ronald c. wek*. (1995). The Histidyl-tRNA Synthetase-Related Sequence in the eIF-2a Protein Kinase GCN2 Interacts with tRNA and Is Required for Activation in Response to Starvation for Different Amino Acids . *Molecular and cellular biology, 15*(08), 4497-4506. 0270-7306/95/$04.00⩽0

Visweswaraiah, J., Lageix, S., Castilho, B. A., Izotova, L., Kinzy, T. G., Hinnebusch, A. G., & Sattlegger, E. (2011). Evidence that eukaryotic translation elongation factor 1A (eEF1A) binds the Gcn2 protein C terminus and inhibits Gcn2 activity. *J Biol Chem, 286*(42), 36568-36579. 10.1074/jbc.M111.248898

5. Appendix

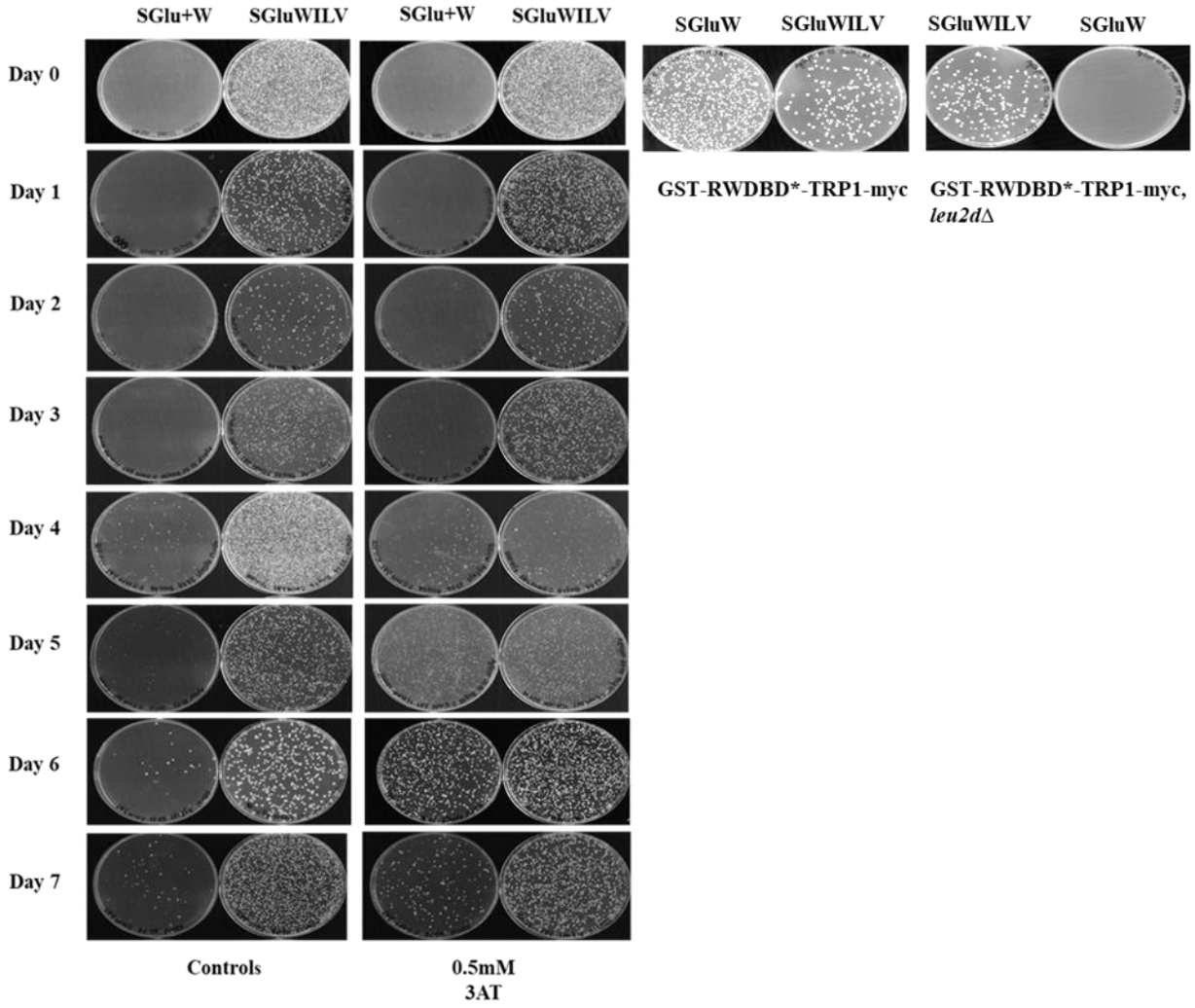


Figure 5. 1 The screening of *LEU2*$^{+}$ *to LEU2*Δ for competitive growth assay culture

The number of colonies were increased as the time of incubation was increased. The SGluW plate supports the growth of *LEU2*$^{+}$ strains whereas SDWILV plate supports growth of both strains.

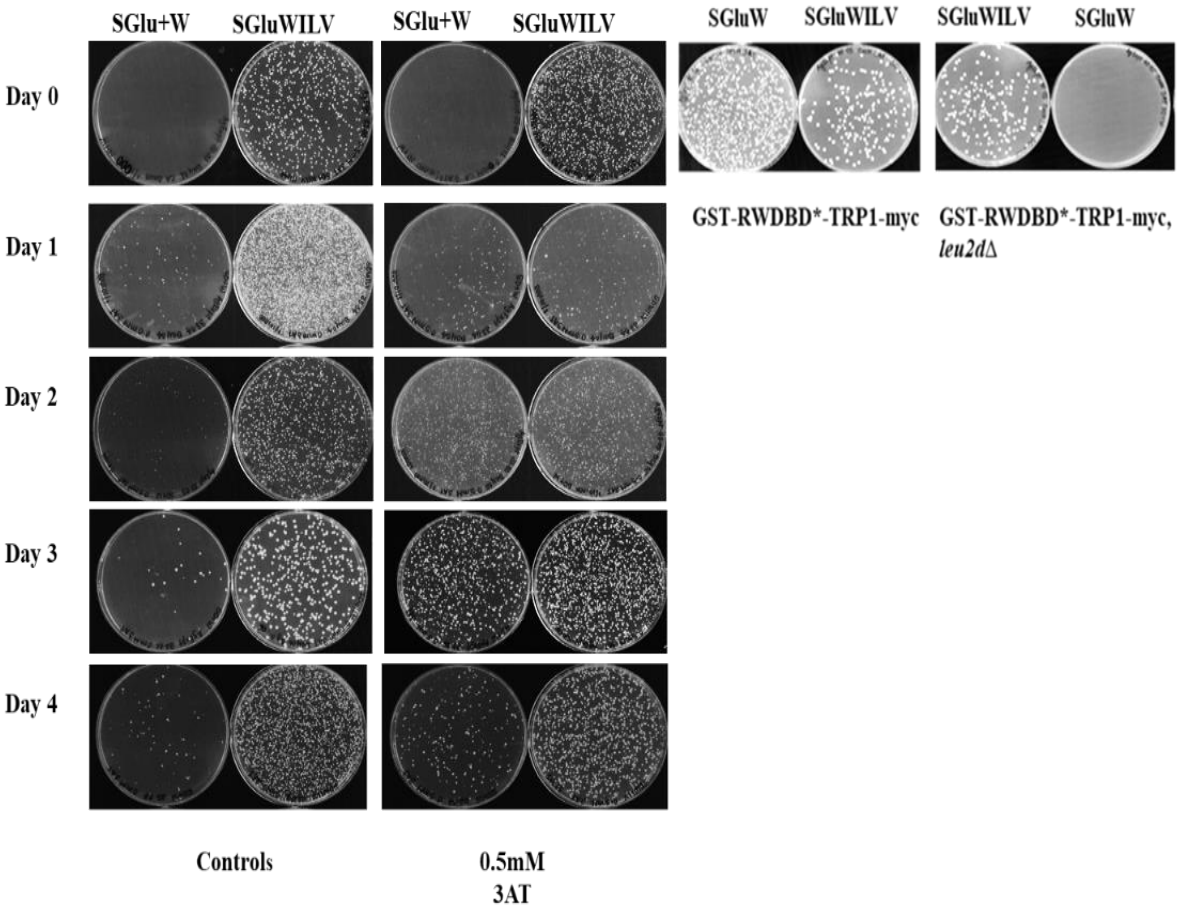


Figure 5. 2 The screening of *LEU2*$^{+}$ *to LEU2*Δ for competitive growth assay culture.

The number of colonies increased as the time of incubation was increased. The SGluW plate supports the growth of *LEU2*$^{+}$ strains whereas SDWILV plate supports growth of both strains.

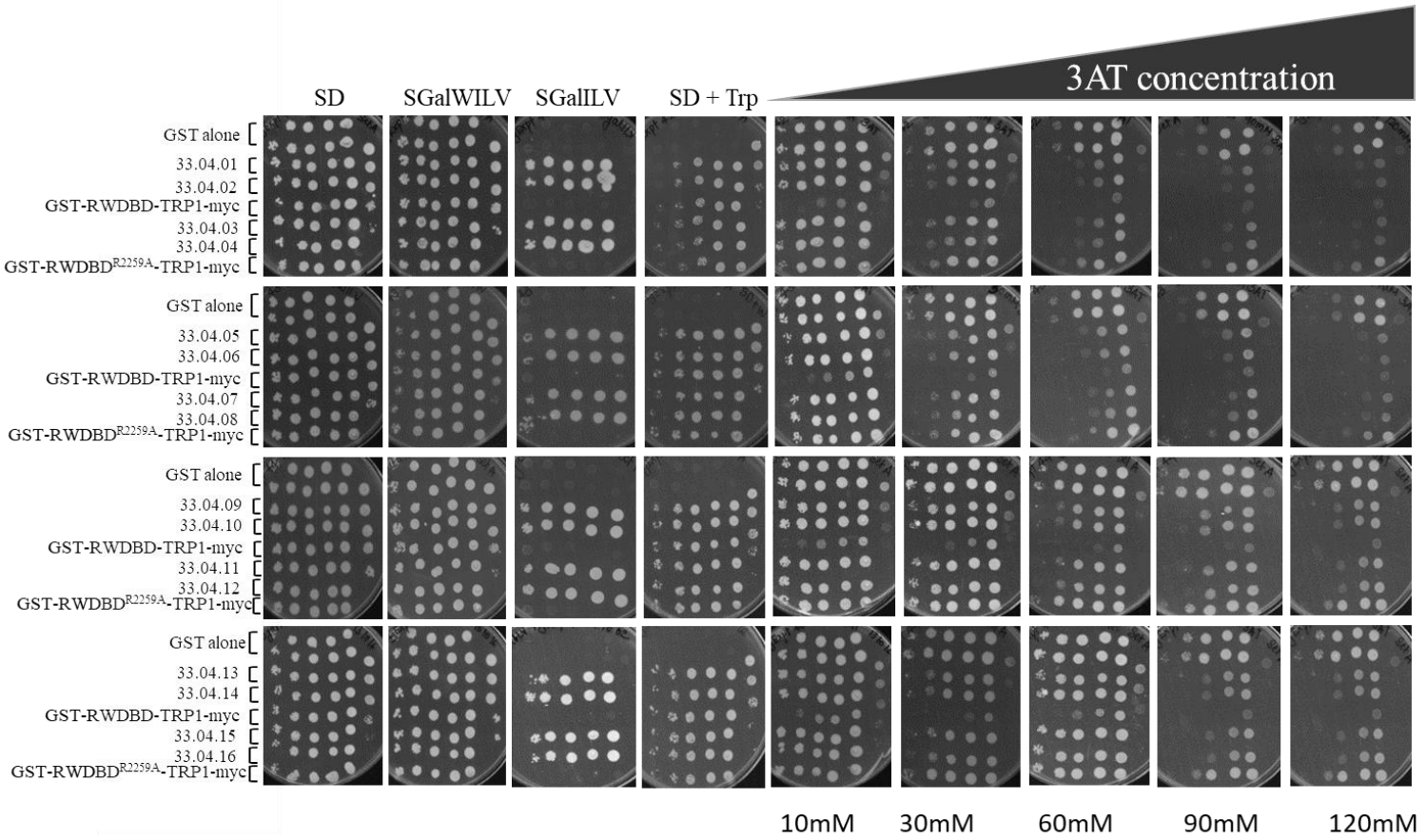


Figure 5. 3 Enriched strains expressed mutated RWDBDs and can overcome the starvation.

5µL of ten-fold serially diluted overnight grown culture of above-mentioned yeast strain expressing were transferred onto solid media along with 3AT. On incubation we showed that, the enriched colonies could overcome the starvation response.

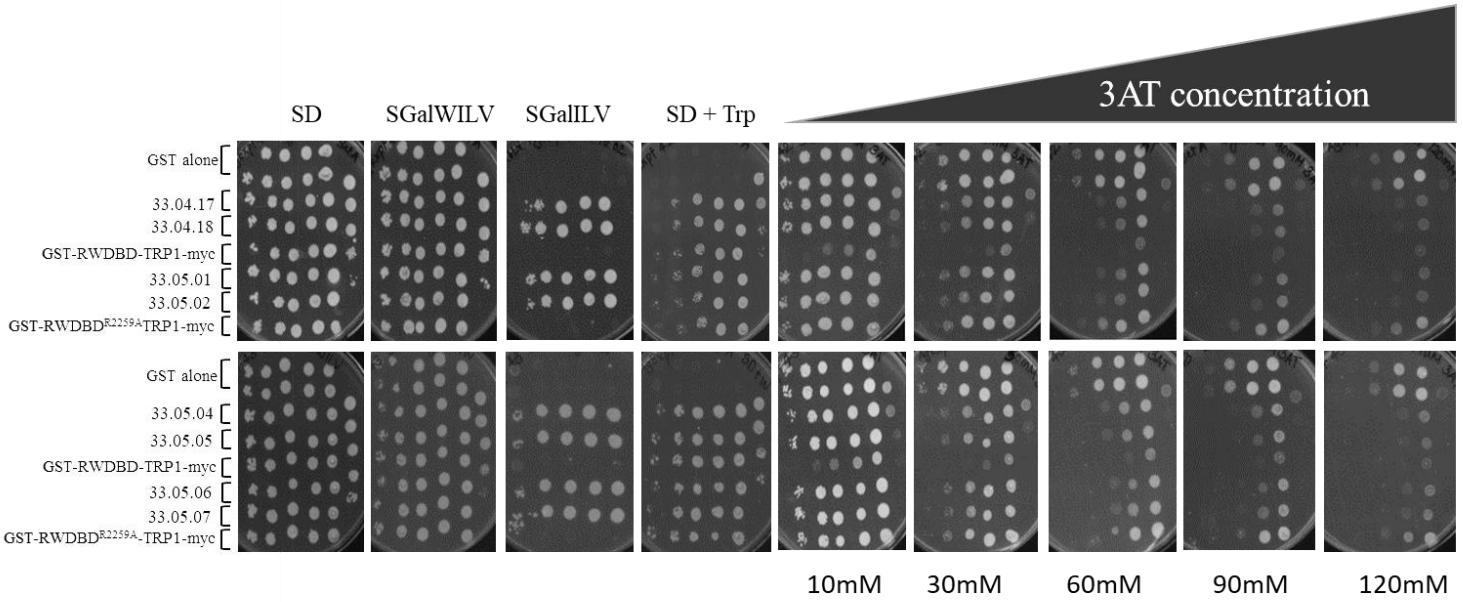

SD
SGalWILV
SGalILV
SD + Trp
3AT concentration
GST alone
33.04.17
33.04.18
GST-RWDBD-TRP1-myc
33.05.01
33.05.02
GST-RWDBD^{R2259A}TRP1-myc
GST alone
33.05.04
33.05.05
GST-RWDBD-TRP1-myc
33.05.06
33.05.07
GST-RWDBD^{R2259A}-TRP1-myc
10mM
30mM
60mM
90mM
120mM

Figure 5. 4 Enriched strains expressed mutated RWDBDs and can overcome the starvation.

5µL of ten-fold serially diluted overnight grown culture of above-mentioned yeast strain expressing were transferred onto solid media along with 3AT. On incubation we showed that, the enriched colonies could overcome the starvation response

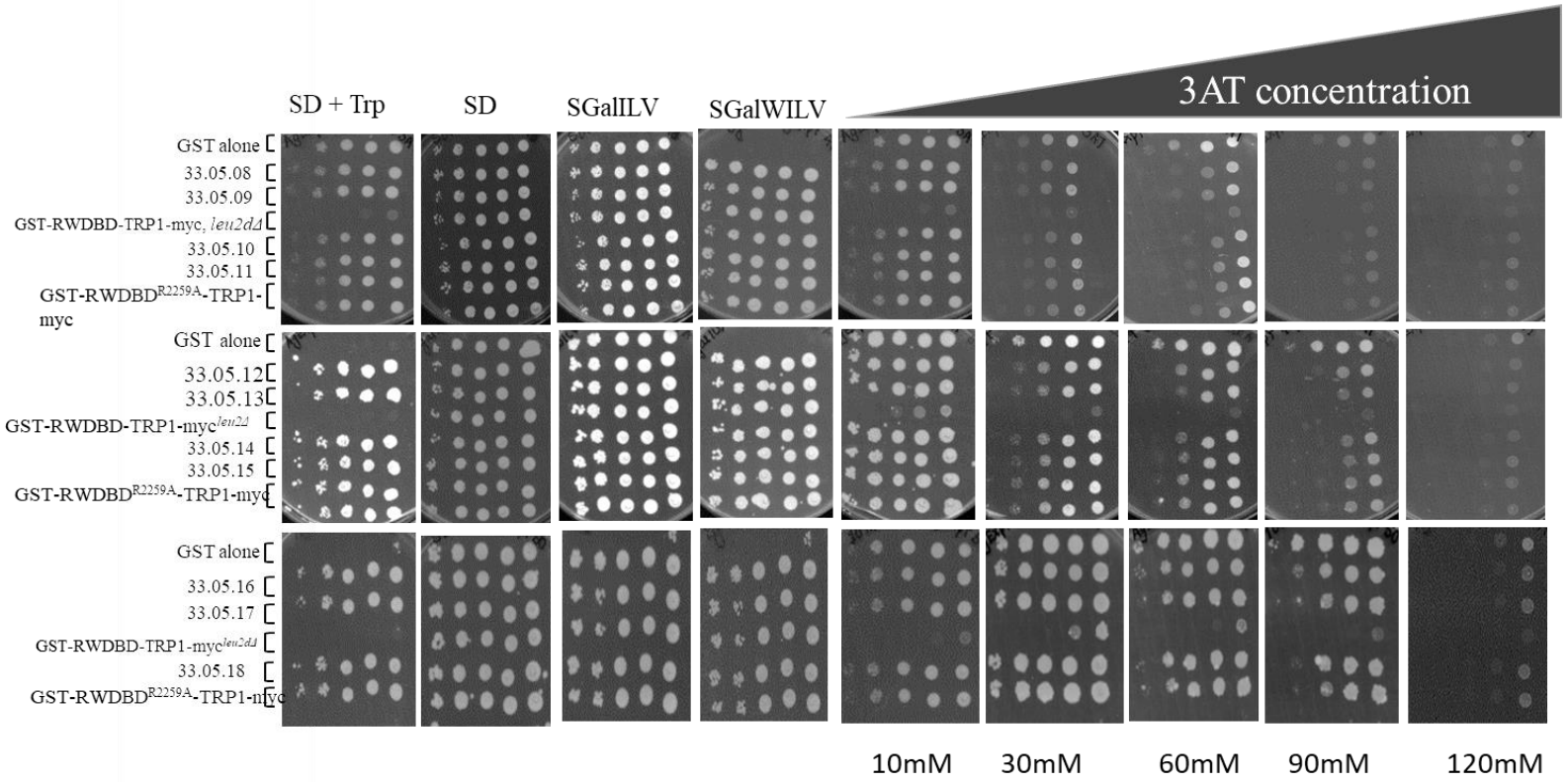


Figure 5. 5 Enriched strains expressed mutated RWDBDs and can overcome the starvation.

5µL of ten-fold serially diluted overnight grown culture of above-mentioned yeast strain expressing were transferred onto solid media along with 3AT. On incubation we showed that, the enriched colonies could overcome the starvation response.

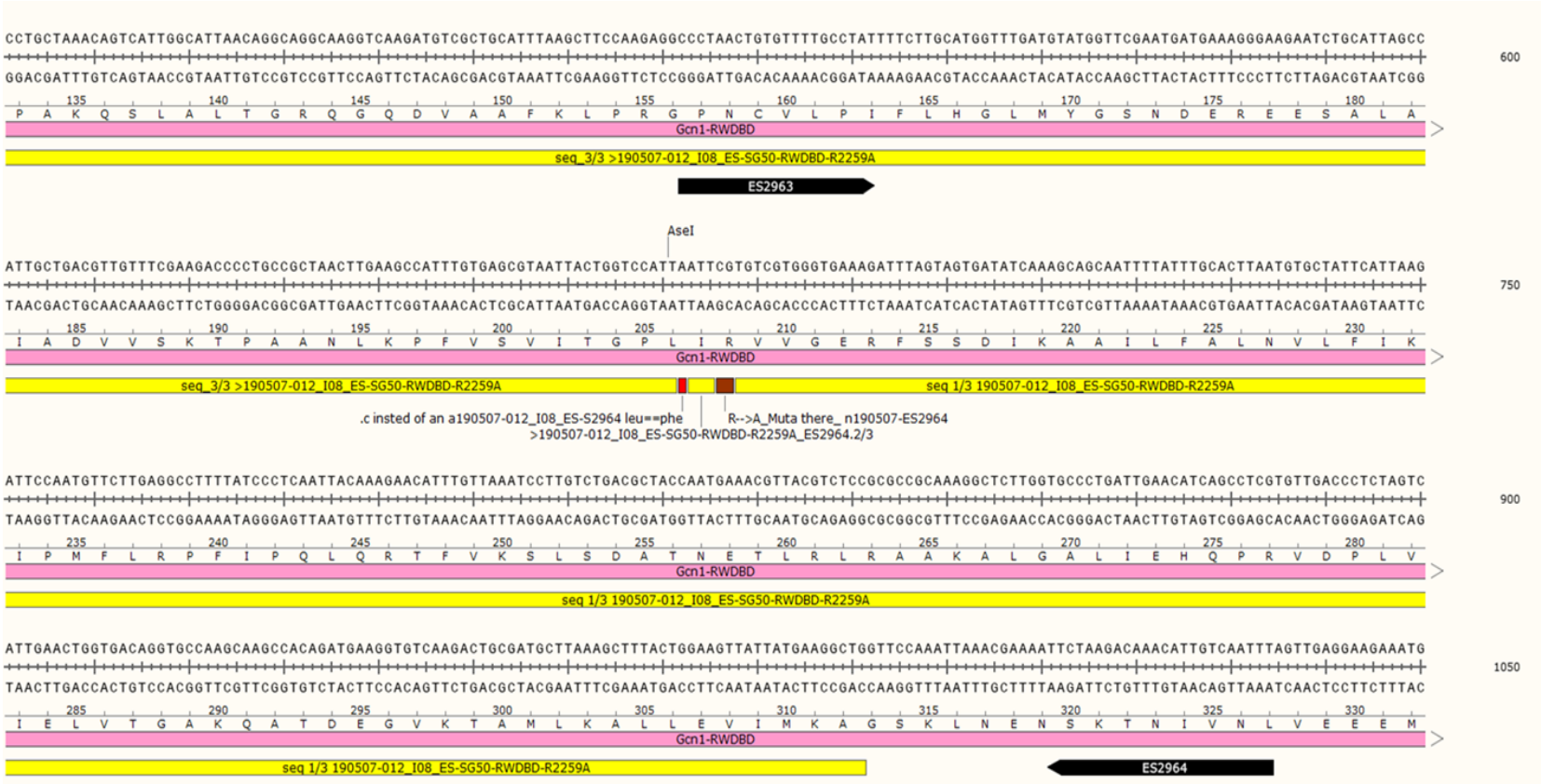


Figure 5. 6 The sequence verification of plasmid pSG50 expressing R2259A-RWDBD domain.

The pink colour Gcn1 denotes yeast genome. The yellow colour denotes sequence was verified sequence through next generation sequencing. The small brown patch observed in the sequence denotes R2259A mutation. The red sequences denote the silent mutation to generate *AseI* restriction site.

Figure 5. 7 The sequence verification of plasmid pSG52 expressing wildtype RWDBD domain.

The pink colour Gcn1 denotes yeast genome. The green colour denotes the sequence was verified sequence through next generation sequencing.

Figure 5. 8 The sequence verification of plasmid pAG01 expressing wildtype RWDBD domain.

The pink colour Gcn1 denotes yeast genome. The green colour denotes the sequence was verified sequence through next generation sequencing.

Table 5. 1 Datapoint for restriction digest screen test.

The below mentioned table shows the datapoint for figure 3.19.

	Mutant (RWDBD-R2259A)	Wildtype
0 hours (0 mM 3AT)	25	75
0 hours (0.5 mM 3AT)	25	75
24 hours (0 mM 3AT)	25	75
24 hours (0.5 mM 3AT)	25	75
48 hours (0 mM 3AT)	25	75
48 hours (0.5 mM 3AT)	100	0
72 hours (0.5 mM 3AT)	100	0
72 hours (0 mM 3AT)	25	75
96 hours (0 mM 3AT)	25	75
96 hours (0.5 mM 3AT)	100	0
120 hours (0 mM 3AT)	25	75
120 hours (0.5 mM 3AT)	100	0
144 hours (0.5 mM 3AT)	100	0
144 hours (0 mM 3AT)	100	0

Table 5. 2 Datapoint for leucine prototrophy test.

The below mentioned table shows the datapoint for figure 3.14.

	LEU2^{+}	*LEU2*Δ
0 hours (0 mM 3AT)	0	*100*
0 hours (0.5 mM 3AT)	0	100
24 hours (0 mM 3AT)	0	100
24 hours (0.5 mM 3AT)	0	100
48 hours (0 mM 3AT)	0	100
48 hours (0.5 mM 3AT)	0	100
72 hours (0.5 mM 3AT)	18	82
72 hours (0 mM 3AT)	0	100
96 hours (0 mM 3AT)	0	100
96 hours (0.5 mM 3AT)	62	38
120 hours (0 mM 3AT)	0	100
120 hours (0.5 mM 3AT)	100	0
144 hours (0 mM 3AT)	8	92
144 hours (0.5 mM 3AT)	84	16

Table 5. 3 Ratio of mutated RWDBD to wildtype RWDBD.

The intensity of bands from Figure 3.16 was measured by ImageJ software. After measuring the intensity of bands data was normalised as mentioned in 2.9. The below table shows the calculative values for Figure 3.18

	3AT con	530 bp	125 bp (multiplied by 4.24)	total	multiply by 100	mutant RWDBD	wildtype RWDBD
0 hours	0	575.142	2438.60208	3013.74408	301374.408	19	81
0 hours	0.5	822.92	3489.1808	4312.1008	431210.08	19	81
24 hours	0	2779.506	11785.10544	14564.61144	1456461.144	19	81
24 hours	0.5	435.971	1848.51704	2284.48804	228448.804	19	81
48 hours	0	918.971	3896.43704	4815.40804	481540.804	19	81
48 hours	0.5	3110.092	0	3110.092	311009.2	100	0
72 hours	0.5	2917.627	0	2917.627	291762.7	100	0
72 hours	0	1084.385	4597.7924	5682.1774	568217.74	19	81
96 hours	0	7650.456	22951.368	30601.824	3060182.4	25	75
96 hours	0.5	1411.92	0	1411.92	141192	100	0
120 hours	0	1442.627	6116.73848	7559.36548	755936.548	19	81
120 hours	0.5	5524.799	0	5524.799	552479.9	100	0
144 hours	0.5	5029.506	0	5029.506	502950.6	100	0
144 hours	0	325.092	0	325.092	32509.2	100	0

Table 5. 4 Datapoint for 0 mM 3AT growth assay.

The mentioned yeast strains were subjected to growth assay. The growth of each strains was measuring every two hours at 600 nm and graph was plotted. The below mentioned table denotes the datapoint for figure 3. 9

Time	WT-GST	GST-Gcn1 del	GST-RWDBD-TRP1-myc	GST-RWDBD*-TRP1-myc
9.15	0.88	0.61	0.11	0.58
11.15	1.14	0.98	0.57	0.98
13.15	2.95	1.51	0.89	1.36
15.15	5.9	3.13	1.57	3.11
17.15	6.7	3.84	1.96	4.17
19.15	7.9	4.32	2.57	5.17

Table 5. 5 Datapoint for 0.025 mM 3 AT growth assay

The mentioned yeast strains were subjected to growth assay. The growth of each strains was measuring every two hours at 600 nm and graph was plotted. The below mentioned table denotes the datapoint for figure 3. 9

Time	WT-GST	GST-Gcn1 del	GST-RWDBD-TRP1-myc	GST-RWDBD*-TRP1-myc
9.15	0.34	0.27	0.16	0.17
11.15	0.53	0.39	0.28	0.32
13.15	1.24	0.41	0.31	0.85
15.15	1.68	0.61	0.61	1.16
17.15	2.39	0.63	0.71	2
19.15	2.98	0.69	0.85	2.61

Table 5. 6 Datapoint for 0.5 mM 3 AT growth assay

The mentioned yeast strains were subjected to growth assay. The growth of each strains was measuring every two hours at 600 nm and graph was plotted. The below mentioned table denotes the datapoint for figure 3. 9

Time	WT-GST	GST-Gcn1 del	GST-RWDBD-TRP1-myc	GST-RWDBD*-TRP1-myc
9.15	0.39	0.21	0.2	0.21
11.15	0.45	0.28	0.35	0.26
13.15	1.19	0.32	0.45	0.88
15.15	1.73	0.48	0.48	1.16
17.15	2.15	0.55	0.54	1.51
19.15	2.73	0.59	0.65	2.3

Table 5. 7 Datapoint for 1 mM 3 AT growth assay

The mentioned yeast strains were subjected to growth assay. The growth of each strains was measuring every two hours at 600 nm and graph was plotted. The below mentioned table denotes the datapoint for figure 3. 9

Time	WT-GST	GST-Gcn1 del	GST-RWDBD-TRP1-myc	GST-RWDBD*-TRP1-myc
9.15	0.16	0.16	0.15	0.21
11.15	0.49	0.2	0.2	0.26
13.15	0.83	0.3	0.28	0.79
15.15	1.47	0.4	0.4	1.37
17.15	1.89	0.44	0.44	1.4
19.15	2.45	0.49	0.48	1.73

Table 5.8 List of reagents used in preparation of solutions and their manufactures.

Reagents	Manufacture name	Country
Agar	FORMEDIUM ™	United Kingdom
Ammonium Persulphate	ACRO ORGANICS	United states of America

EDTA	SIGMA™	United states of America
Lithium Acetate	SIGMA™	United states of America
PEG P4338	SIGMA™	United states of America
Potassium Chloride	SIGMA™	United states of America
MgCl2 Hexahydrate	SIGMA™	United states of America
Ampicillin	FORMEDIUM ™	United Kingdom
Ethidium Bromide	SIGMA™	United states of America
Yeast Extract	FORMEDIUM ™	United Kingdom
Tris Base	FORMEDIUM ™	United Kingdom
Tryptone	FORMEDIUM ™	United Kingdom
Agarose	FORMEDIUM ™	United Kingdom
Galactose	FORMEDIUM ™	United Kingdom
Glucose	FORMEDIUM ™	United Kingdom
YNB without amino acids	FORMEDIUM ™	United Kingdom
Peptone	FORMEDIUM ™	United Kingdom
$CaCl_2$	SIGMA	United states of America
Isoleucine	FORMEDIUM ™	United Kingdom
leucine	FORMEDIUM ™	United Kingdom
Valine	FORMEDIUM ™	United Kingdom

Tryptophan	FORMEDIUM ™	United Kingdom
3AT	FORMEDIUM ™	United Kingdom
$Nacl_2$	SIGMA	United states of America