Function of Gcn4 during the unfolded protein response

CAITLIN DOREEN GOULD

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Abstract

Endoplasmic reticulum (ER) stress, resulting from an accumulation of misfolded proteins, can induce a mechanism termed the unfolded protein response (UPR). In *Saccharomyces cerevisiae*, it is the inositol-requiring 1 (Ire1) pathway that is implicated in the UPR to restore cellular homeostasis. Upon activation, Ire1 splices *HAC1* mRNA to produce *HAC1*^{i} (induced), which is translated into the protein Hac1. This basic leucine zipper (bZIP) transcription factor binds to UPR-associated genes that contain an unfolded protein response element (UPRE), ultimately initiating transcription in response to ER stress. Hac1 exists as a bZIP transcription factor that is traditionally thought to bind to UPREs as a homodimer. Previous work has however suggested that Hac1 may exist as a heterodimer with another bZIP transcription factor, general control nonderepressible 4 (Gcn4), which is activated by general control nonderepressible 2 (Gcn2). Gcn4 is conventionally associated with the general amino acid control (GAAC) and is produced under an imbalance of amino acids. This study aimed to explore the requirement of Gcn4 in the UPR. Under a balanced provision of amino acids, *GCN2* and *GCN4 S. cerevisiae* deletion strains did not show a decrease in expression of a *KAR2-lacZ* reporter from a Z691 plasmid. Under an imbalance of amino acids, both deletion strains exhibited reduced activity of this reporter. However, after correction for translational efficiency using a *GCN4Δ4uORF-lacZ* reporter from a p227 plasmid, no difference was observed, suggesting Gcn2 and Gcn4 have no role in the UPR. This work could therefore have importance in supporting previous studies that suggest that Hac1 and Gcn4 have distinct roles in different regulatory pathways within *S. cerevisiae*.

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Master in Research (MRes) Thesis

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1.0 **Statement of Copyright**

2.0 **Acknowledgements**

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3.0 **Dedication**

For my Mam, who always believed in me.

4.0 **Introduction**

4.1 Role of the endoplasmic reticulum

An organelle that has distinct morphologies is the endoplasmic reticulum (ER). The ER is divided into the smooth ER (SER) and rough ER (RER), consisting of tubules and sheets respectively (Shibata *et al*., 2010). In eukaryotes, the RER is structurally connected to the nuclear envelope, and has ribosomes bound to its cisternae (Shibata *et al*., 2010; Voeltz *et al*., 2006). Therefore, secretory proteins produced by the 80 S membrane-bound ribosomes can co-translationally translocate into the RER via a protein-conducting channel (PCC) (Beckmann *et al*., 2001). Once nascent proteins enter the RER luminal domain, they can be assisted in folding by the binding of molecular chaperones and interaction of folding enzymes, which can form disulphide bonds during folding (Chen *et al*., 1995; Laboissière *et al*., 1995). Additional post-translational modifications can also occur in the ER, which can include oligosaccharyltransferase (OST) catalysed N-linked glycosylation (Ruiz-Canada *et al*., 2009).

4.2 Protein misfolding and ER stress responses

Despite the maintenance of protein folding via the mechanisms previously mentioned (Chen *et al*., 1995; Laboissière *et al*., 1995; Ruiz-Canada *et al*., 2009), this process of folding proteins can however be disrupted, resulting in ER stress. This resultant stress from an accumulation of misfolded proteins can lead to cytosolic ER-associated protein degradation (ERAD) or ER-to-lysosome-associated degradation (ERLAD), in compensation of

ERAD (Fasana *et al*., 2024; McCracken and Brodsky, 1996). Furthermore, the unfolded protein response (UPR) is also activated in response to stress in the ER (Kimata *et al*., 2006). Both ERAD and UPR pathways are required to maintain the viability of the cell, and more specifically the UPR to reduce oxidative damage and protein translation via the up-regulation of genes that confer resistance to oxidative stress, and down-regulation of genes that would translate into nascent proteins (Kimata *et al*., 2006; Travers *et al*., 2000).

4.3 The UPR and its associated pathways

The UPR can have outcomes including the maintenance of cell survival via gene regulation and the production of molecular chaperones, such as the glucose-regulated protein (GRP) and immunoglobulin heavy chain binding protein (BiP) GRP78 (Kimata *et al*., 2006; Kozutsumi *et al*., 1988; Lin *et al*., 2007; Liu *et al*., 2023). Alternatively, apoptosis can occur if the distinct UPR signalling pathways (PERK, ATF6 and Ire1) are diminished through constant activation under ER stress (Lin *et al*., 2007). These pathways include the RNA-activated protein kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring 1 (Ire1) ER cell surface receptors in mammalian cells (Hamid *et al*., 2020; Harding *et al*., 1999; Okada *et al*., 2002).

4.3.1 PERK in the UPR

ER stress can activate the type-1 transmembrane protein PERK in the ER. PERK contains two domains, the cytosolic and luminal domain (Harding *et*

al., 1999). The cytosolic (kinase) domain is structurally comprised of two lobes, the C-terminal lobe (C-lobe) and N-terminal lobe (N-lobe), with a hinge loop connecting them both (Cui *et al*., 2011). The highly abundant molecular chaperone BiP is expressed more during the UPR (Bakunts *et al*., 2017; Morris *et al*., 1997). BiP interacts with the luminal domain of PERK via binding to its ATPase domain. This is important in PERK signalling, as the luminal domain of this sensor cannot detect misfolded proteins. Therefore, BiP is required to detect ER stress by binding to misfolded proteins in its substrate binding domain (Carrara *et al*., 2015). The dissociation of BiP from PERK results in the oligomerisation and subsequent phosphorylation of PERK monomers (Ma *et al*., 2002). This activation then leads to the phosphorylation of eukaryotic initiation factor 2 α (eIF2α) at serine 51 residue (Harding *et al*., 1999). This phosphorylation inhibits the change of GDP to GTP, which originally occurs via eIF2B, which is an exchange factor for nucleotides. Therefore, translation of proteins is hindered as methionyl initiator tRNA (Met-tRNA$_i$) cannot be delivered to the 40 S subunit of the ribosome to initiate translation (Kapp and Lorsch, 2004; Pavitt *et al*., 1998). The translation of activating transcription factor 4 (*ATF4*) mRNA can also occur as a result of eIF2α phosphorylation, resulting in the expression of the genes *GADD34* and *CHOP* in mammalian cells. This is due to *ATF4* now having the correct translation initiation site that would not be available in non-stressed cells, as ribosomes take an extended period of time to begin translation again. Therefore, the ribosomes can reinitiate translation at the *ATF4* mRNA, as by this time they have already bypassed the inhibitory second upstream open reading frame (uORF) for *ATF4* (Harding *et al*., 2000; Ma and Hendershot, 2003; Vattem and Wek, 2004). *GADD34* is important in this signalling

pathway, as growth arrest and DNA damage-inducible protein ($GADD_{34}$) dephosphorylates eIF2α by localising type 1 protein serine/threonine phosphatase 1 (PP_1) to the ER; translation can then reinitiate following dephosphorylation (Brush *et al*., 2003; Ma and Hendershot, 2003). However, apoptosis can alternatively occur via the induction of *CHOP*. C/EBP homologous protein (CHOP) can increase the translation of proteins before cellular homeostasis is restored, creating reactive oxygen species (ROS) and decreasing levels of ATP, resulting in apoptosis (Han *et al*., 2013).

4.3.2 ATF6 in the UPR

Another signalling pathway in the UPR involves ATF6, which is a type-2 transmembrane protein, with a luminal C-terminal and cytosolic N-terminal. ATF6 in mammalian cells contains two proteins, ATF6α and ATF6β. Both proteins contain a basic-leucine zipper (bZIP) domain and DNA-binding domain. However, only ATF6α has a VN8 sequence (8 amino acids) in its N-terminal, which allows this protein to induce transcription more frequently (Haze *et al*., 1999; Haze *et al*., 2001; Thuerauf *et al*., 2002). ATF6 similar to PERK, has BiP interacting with it, and dissociation of BiP occurs upon the sensing of ER stress (Carrara *et al*., 2015; Shen *et al*., 2005). This dissociation reveals golgi localisation signals (GLSs) at two regions in ATF6's luminal domain, meaning ATF6 can be exported out of the ER to the golgi apparatus via the packaging of ATF6 into vesicles by a coat protein complex II (COPII) of proteins (Kuehn *et al*., 1998; Schindler and Schekman, 2009; Shen *et al*., 2002). In the golgi, ATF6 is cleaved by site-1 protease (S1P) at an RxxL motif (x is any amino acid) and site-2

protease (S2P) at asparagine and proline in the ATF6 sequence (Ye *et al*., 2000). The N-terminal (NH_2) of ATF6 can then enter the nucleus, forming a complex with the transcription factor human nuclear factor (NF-Y) (CCAAT binding factor (CBF)), creating an ER stress response factor (ERSF), which induces transcription of genes at ER stress response elements (ERSEs) such as *GRP78* to alleviate ER stress (Haze *et al*., 1999; Roy *et al*., 1996; Yoshida *et al*., 2000).

4.3.3 Ire1 in the UPR

An additional type-1 transmembrane protein that is implicated in the UPR is Ire1 (Cox *et al*., 1993). Ire1 is a UPR signalling protein that is evolutionarily conserved, as it is found in *Saccharomyces cerevisiae* in addition to mammalian cells (Cox *et al*., 1993; Li *et al*., 2010). Similar to PERK, Ire1 contains a C-terminal situated in the cytosol. This C-terminus contains an endoribonuclease (RNase) domain and kinase domain. Additionally, it has an N-terminal that is located in the ER lumen, which KAR2 (BiP in mammalian cells) interacts with (Mori *et al*., 1992; Pincus *et al*., 2010; Poothong *et al*., 2010). When ER stress is detected, the molecular chaperone dissociates from Ire1 and binds to unfolded proteins in the ER. This dissociation causes two Ire1 monomers to oligomerise and *trans*-autophosphorylate (Okamura *et al*., 2000). Once activated, Ire1 splices an uninduced form of *HAC1* (*HAC1*^{u}) mRNA. The spliceosome is not required for splicing of *HAC1* mRNA, instead Ire1 uses the RNase domain located in its C-terminus on the 5' and 3' splice junctions of the mRNA of *HAC1* (Poothong *et al*., 2010; Sidrauski and Walter, 1997). An intron of 252 nucleotides is subsequently removed, leaving a 5' exon and a

3’ exon (Cox and Walter, 1996). The 5’ exon is left with a 2’,3’-cyclic phosphate at the spliced 3’ end, and the 3’ exon is left with a 5’ hydroxyl group at the spliced 5’ end. The exposed ends of these exons are held in position by base pairing to be ligated by yeast tRNA ligase (Rlg1/Trl1) (Gonzalez *et al*., 1999; Sidrauski *et al*., 1996). This induced form of *HAC1* (*HAC1*^{i}) mRNA is translated into the protein homologous to ATF/CREB 1 (Hac1), a CRE-motif binding protein, via the reactivation of ribosomes along the mRNA (Nojima *et al*., 1994; Rüegsegger *et al*., 2001).

Alternatively in mammalian cells, Ire1 exists as an Ire1α homologue and an Ire1β homologue. Ire1α exhibits a higher splicing activity of the mRNA for X-box binding protein (*XBP1*^{u}) than Ire1β, removing an intron of 26 nucleotides from the mRNA (Imagawa *et al*., 2008; Lee *et al*., 2002; Tirasophon *et al*., 2000). *XBP1*^{s} is then produced by ligation of the 2’,3’-cyclic phosphate and the 5’ hydroxyl group exon termini by the tRNA ligase complex, with archease and RTCB forming this complex, exhibiting splicing activity (Jurkin *et al*., 2014; Lu *et al*., 2014). The protein XBP1 is produced and alike Hac1, acts as a transcription factor (Mori *et al*., 1998; Yoshida *et al*., 2001).

Hac1 can bind to a set of genes specifically, as they contain an unfolded protein response element (UPRE) with a palindromic sequence, CAGCGTG, separated by a cytosine nucleotide (Mori *et al*., 1998). Such genes include *KAR2*, which encodes the orthologue of the mammalian molecular chaperone BiP (Mori *et al*., 1992). In mammalian cells, XBP1 also acts as a transcription factor by binding to ERSEs, such as ERSE-26,

with the sequence CCAAT-N26-CCACG. This promoter is present in genes such as *ERLEC1/XTP3-B*, encoding a lectin associated with the ER, which is implicated in ERAD (Hosokawa *et al*., 2008; Misiewicz *et al*., 2013). This shows the interconnection between the UPR and ERAD pathways and the role of the bZIP transcription factors Hac1 and XBP1 in inducing genes associated with these pathways (Zhang *et al*., 2016).

4.4 bZIP transcription factors

As previously stated, the bZIP transcription factors Hac1 and XBP1 have a role in the UPR and ERAD, via binding to UPREs and ERSEs to activate genes associated with these pathways (Misiewicz *et al*., 2013; Mori *et al*., 1998). bZIP transcription factors are conserved between eukaryotes and have structures that are very similar between them (Jindrich and Degnan, 2016). They are comprised of two monomers each with a basic region made of an α-helix. This region forms a coiled-coil, which interacts with half-binding sites of DNA on its major groove. Binding of the bZIP's fork region to the DNA, created by the separation of the coiled-coil facilitates this (Ellenberger *et al*., 1992). The leucine zipper region of these proteins is characterised by a sequence of amino acids that have a leucine spaced at intervals of seven amino acids repeated in the sequence (LxxxxxxLxxxxxxL) (Lively *et al*., 2004). In this sequence, the seven amino acids are termed a heptad of **a, b, c, d, e, f** and **g**. The **a** position of amino acids in the leucine zipper region can determine the dimerisation specificity of bZIP transcription factors. Homodimers can form from amino acids such as asparagine in this position and heterodimers can form from

this amino acid coupled with lysine for example, at the **a**-**a** interface (Acharya *et al*., 2002).

bZIP proteins also exhibit preferences when binding as homodimers or heterodimers based on which subclass they are divided into. In *S. cerevisiae*, the previously mentioned protein Hac1 from the ATF/CREB family, has the preference of binding as a homodimer. Other homodimer-favouring bZIP transcription factors that are not in the same sub-class also exist, for example general control nonderepressible 4 (Gcn4) from the AP-1 family that can activate in response to a depletion of amino acids (Deppmann *et al*., 2006; Ellenberger *et al*., 1992; Hope and Struhl, 1985; Nojima *et al*., 1994).

4.5 The general amino acid control

In yeast, the bZIP transcription factor Gcn4 has been found to bind to the promoters of biosynthetic genes of amino acids, such as *HIS3* and *TRP5*, during the general amino acid control (GAAC) (Hope and Struhl, 1985). This pathway is activated upon limitation of a range of amino acids, leading to an abundance of tRNA that is uncharged. General control nonderepressible 2 (Gcn2) can detect this change by binding of the uncharged tRNA to the histidyl-tRNA synthetase (HisRS) and C-terminal binding segment (C-term) of this kinase. Gcn2 upon binding of these uncharged tRNAs can phosphorylate eIF2α (Dong *et al*., 2000; Wek *et al*., 1995). This in turn leads to the translation of *GCN4*, which has four uORFs that under normal cellular conditions, repress the translation of the *GCN4* mRNA. *GCN4* is repressed as ribosomes translate the first uORF, then initiate again at the fourth uORF by the 40 S subunit interacting with

eIF2·GTP·Met-tRNA$_i$ ternary complexes. However, during starvation there is a depletion of these complexes, meaning scanning between the length of the first and fourth uORF occurs because ribosomes have not bound to the ternary complexes. The ribosomes will then be able to reinitiate translation at the point between the fourth uORF and *GCN4*, as translation has been slowed, meaning the Gcn4 protein will be translated (Dever *et al*., 1992; Mueller and Hinnebusch, 1986). Gcn4 can then act as a transcription factor to activate amino acid biosynthetic genes associated with the general control pathway to counteract starvation (Hope and Struhl, 1985).

4.6 Homodimerisation of Hac1 during the UPR

It has been established that the transcription factor Hac1 binds to UPREs of target genes during the UPR in *S. cerevisiae*. *In vitro* studies using microfluidic affinity analysis MITOMI 2.0 has characterised that there are two UPRE binding sites, extended core (xc) UPRE-1, which has been found to be 11 to 12 base pairs (bp) long and requires flanking sequences to be sufficient in binding to Hac1. Alternatively, a second UPRE has been characterised to be shorter at 6 to 7 bp, with more binding of the induced form of Hac1 to UPRE-2 and was shown to have a higher level of transcriptional activity when the UPR was induced in *S. cerevisiae* (Fordyce *et al*., 2012). In addition to this finding, the study also supports the idea that Hac1 exists as a homodimer because the 6X-His tagged Hac1 that was used, was created by flowing the *in vitro* translation system over an anti-His antibody coated surface. This was able to collect purified Hac1, and due to this Hac1 being produced this way, was most likely to exist as either monomers of Hac1 or homodimers produced from Hac1 monomers.

Homodimers of Hac1 have also been proposed from earlier studies, in which Hac1 (termed Ern4p in the study) was deleted from yeast cells (*ern4*Δ). The result of this deletion was the inability of these cells to transcriptionally induce stress genes localised to the ER, such as *KAR2* and *PDI1*. Therefore, suggesting Hac1 exists as a homodimer, as it is required to induce these target genes (Mori *et al*., 1996). Furthermore, this study suggests that a homodimer of Hac1 exists, as suggested from the electrophoretic mobility shift assays that were performed. Hac1 was expressed in and purified from *Escherichia coli* in these assays, meaning that this purified protein existed on its own. Therefore, this shows from the results of the study that Hac1 sufficed in binding to WT and mutated UPREs *in vitro*, without the requirement of another transcription factor.

4.7 Heterodimerisation of Hac1 and Gcn4 during the UPR

Alternatively, Hac1 has been proposed to form a heterodimer with Gcn4, the bZIP transcription factor that activates amino acid biosynthetic genes during the GAAC (Hope and Struhl, 1985; Patil *et al*., 2004). This study conducted by Patil *et al* (2004) had found from bioinformatic analysis, that the core UPRE was not present in the promoters of a large majority of target genes of the UPR. Furthermore, from this analysis they grouped target gene promoters that had sequences which were the most similar into motifs, discovering novel UPRE-2 and UPRE-3 from motif 1 (TACGTG) and motif 8 (AGGACAAC) respectively. The results from this analysis suggested that other transcription factors that bind to the novel UPREs exist in addition to Hac1. Through investigating this, Gcn4 was discovered as being able to

bind to UPRE-2 independent of Hac1 in cells depleted of amino acids when overexpressed. Furthermore, it was also found that in not only UPRE-2, but also the core UPRE and UPRE-3, that during ER stress, Gcn4 was required. Cells deleted for Gcn4 (Δ*gcn4*) and its activator Gcn2 (Δ*gcn2*) proved this, as they could not mount a transcriptional response under stress conditions. The requirement of Gcn4 during the UPR in this study also suggests that it either exerts its activity downstream of Hac1, or it alternatively acts with it because no transcription was induced in Δ*gcn4* cells at all three UPREs in contrast to wild-type (WT) cells that both Hac1 and Gcn4 were present in. A supershift analysis was lastly carried out to confirm that both Hac1 and Gcn4 bind together at two distinct UPREs, suggesting a heterodimer forms. The proposed mechanism of this heterodimer formation is Gcn4 is maintained by Gcn2 at a basal level in cells which have no Hac1 present. However, when the UPR is elicited, the synthesis of Hac1 occurs via Ire1 splicing and it binds to UPREs in target genes. This study suggests however that Hac1 cannot induce transcription alone and that Gcn4 is stabilised via interacting with Hac1, which then leads to transcriptional induction by a ternary complex of Hac1, Gcn4 and the DNA of the promoter region (Fig 1).

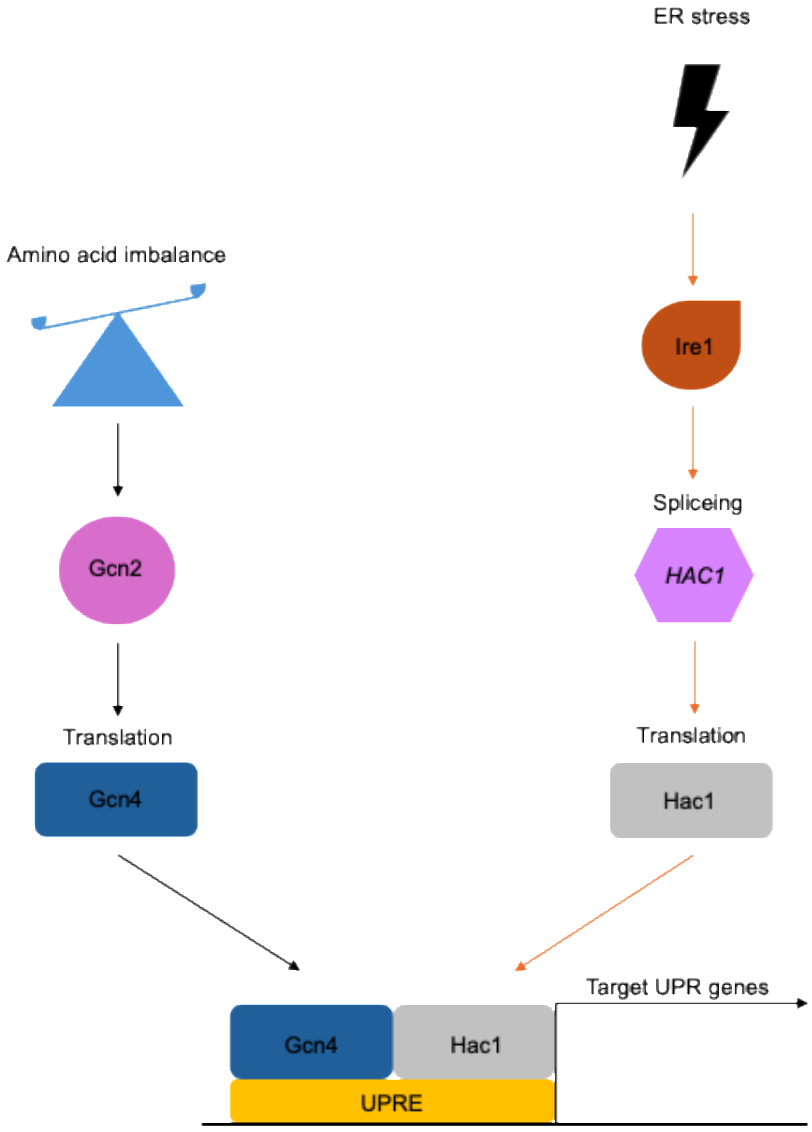

Fig 1. Schematic of possible homologous to ATF/CREB 1 (Hac1) and general control nonderepressible 4 (Gcn4) heterodimerisation to upregulate target unfolded protein response (UPR) genes in *Saccharomyces cerevisiae*. Black arrows represent the general amino acid control (GAAC). An amino acid imbalance causes general control nonderepressible 2 (Gcn2) to activate the translation of Gcn4. Orange arrows represent the UPR. ER stress causes the activation of inositol-requiring 1 (Ire1), which splices *HAC1* mRNA, that is translated to Hac1. It is proposed that Gcn4 maintained at a basal level is stabilised by Hac1, which is produced as a result of the UPR. Both Gcn4 and Hac1 bind to unfolded protein response elements (UPREs), resulting in transcription.

While suggesting that a heterodimer of Hac1 and Gcn4 forms, the Patil study may also have inadvertently recapitulated the role of Gcn4 in the GAAC. This is possible as the WT strain used in this study had the genotype *MAT a; ura3-1; leu2-3,-112; his3-11,-15; trp1-1; ade2-1; can1-100*, that supplementation of media with amino acids, such as leucine, was required for growth. In doing this, the GAAC can be activated if other branched amino acids, isoleucine and valine are not supplemented with leucine. Due to these amino acids each being involved in the biosynthesis of each other, supplementation of one amino acid and not the others will lead to an imbalance within the cell causing the GAAC to become activated (KEGG, 2021; Wek *et al*., 1995). It can be suggested that this had occurred from the results of the Patil study, as their results showed that in WT cells, there were higher levels of both eIF2α phosphorylation and Gcn4 tagged with myc compared to Δ*gcn2* cells. Thus, showing that the GAAC was activated, as only the WT cells containing both Gcn2 and therefore Gcn4 could amount a response to ER stress. This interpretation of the results of the Patil study suggests an indirect role of Gcn4 during the UPR, suggesting that it may be required to correct an amino acid imbalance and not bind as a heterodimer with Hac1. This indirect role for Gcn4 is important in terms of the UPR, as if an amino acid imbalance is present, proteins cannot be synthesised, due to a scarcity of amino acids. This therefore suggests that the UPR cannot be activated without Gcn4, as it requires the translation of proteins produced from amino acids (Schröder, unpublished).

4.8 Aims and objectives

From these interpretations, they raise the question of what the function of Gcn4 is during the UPR in the transcriptional induction of target genes? Therefore, this present study aimed to establish during the UPR, what the role of Gcn4 is and whether it has an indirect role in this pathway through activating the GAAC. The main objectives of this study thus were to construct novel *S. cerevisiae* strains with Gcn2 and Gcn4 deleted. These strains were prototrophic for all 20 proteinogenic amino acids, meaning amino acid supplementation was not required, avoiding possible undesired activation of the GAAC. Furthermore, the WT, *gcn2*Δ and *gcn4*Δ strains were to be transformed with reporter plasmids to observe their differences in activity during the UPR and GAAC. To carry this out, protein and. β-galactosidase concentrations were to be measured and compared for each strain transformed with each reporter plasmid. The conditions in which these strains were to be grown in were a balance of amino acids vs an imbalance of amino acids to elicit the GAAC. Dithiothreitol (DTT) was used to elicit the UPR in all strains transformed with a reporter plasmid designed to measure the response of this pathway.

If the hypothesis of Gcn4 having an indirect requirement during the UPR under an imbalance of amino acids is correct, then the results should show a decrease in activity in *gcn2*Δ and *gcn4*Δ strains compared to the WT strain, as the GAAC cannot be activated without Gcn2 and Gcn4. To further confirm this, the results of experiments performed under a balance of amino acids should display the same activity in all three strains, as the GAAC

should not be activated under this nutritional supply, meaning Gcn2 and Gcn4 will not be required to elicit a response.

5.0 **Materials and Methods**

5.1 Materials

5.1.1 Buffers, stock and specialist solutions

All buffers that required preparation prior to use in this project are displayed in Table 1, along with their accompanying preparations to make the required volume. Buffers requiring autoclaving were done so at 121 °C for 20 min. Stock solutions used that did not require preparation or were commercially available are stated throughout the Methods section. All other solutions required for this project are displayed in Table 2, with their volume and preparation. Autoclaving of these solutions was again done at 121 °C for 20 min.

Table 1. Preparation of buffers.

Buffer	**Volume (ml)**	**Preparation**
1 X TAE	1000	20 ml 50 X TAE (provided)
		Make to 1000 ml with filtered H_2O
1 X TE, pH 8.0	50	5 ml 10 X TE, pH 8.0 (provided)
		Make to 50 ml with sterile H_2O

Table 2. Preparation of solutions.

Solution	Volume (ml)	Preparation
2 mM dNTPs	1	10 µl 100 mM Tris·HCl, pH 8.0
		20 µl 100 mM dATP
		20 µl 100 mM dCTP
		20 µl 100 mM dGTP
		20 µl 100 mM dTTP
		910 µl sterile H_2O
70% (v/v) EtOH	250	175 ml 100% EtOH
		75 ml sterile H_2O
50% (v/v) EtOH	250	125 ml 100% EtOH
		125 ml sterile H_2O
10% 3 M NaOAc, pH 6.0	100	10 g NaOAc·$3H_2O$
		Use ~ 60 ml filtered H_2O to dissolve
		Use glacial HOAc to adjust to pH 6.0
		Make to 100 ml with filtered H_2O
		Autoclave
1 M LiOAc	250	25.5 g LiOAc·H_2O
		Use ~ 200 ml filtered H_2O to dissolve
		Make to 250 ml with filtered H_2O
		Filter sterilise
50% (w/v) PEG 4000	500	250 g PEG 4000
		200 ml filtered H_2O
		Make to ~ 450 ml with filtered H_2O and mix well
		Make to 500 ml with filtered H_2O
		Autoclave
1-step buffer	10	2 ml 1 M LiOAc
		8 ml 50% (w/v) PEG 4000
30% (v/v) glycerol	500	189 g glycerol
		Make to ~ 400 ml with filtered H_2O and mix well
		Make to 500 ml with filtered H_2O
		Autoclave
50 mM EDTA	500	9.31 g $Na_2EDTA·2H_2O$
		Use ~ 350 ml filtered H_2O to dissolve
		Use 10 M NaOH to adjust to pH 8.0
		Make to 500 ml with filtered H_2O
		Autoclave
50% (v/v) 2-propanol	100	50 ml 2-propanol
		50 ml sterile H_2O
1 N perchloric acid	2.5	231 µl 65% perchloric acid
		2.27 ml sterile H_2O

1.6% (v/v) acetaldehyde	25	~ 500 µl 99.5% (w/w) acetaldehyde in weighed 50 ml tube
		Use ~ 5 ml sterile H_2O to dissolve
		Re-weigh tube
		Use sterile H_2O to adjust to 1.6% (v/v) acetaldehyde concentration
Diphenylamine reagent	5	75 mg diphenylamine
		Use 5 ml glacial HOAc to dissolve
		75 µl concentrated H_2SO_4
		25 µl 1.6% (v/v) acetaldehyde
1 M dithiothreitol (DTT)	10	1.54 g dithiothreitol
		Use ~ 9 ml filtered H_2O to dissolve
		Make to 10 ml with filtered H_2O
		Filter sterilise
1 M Na_2CO_3	500	53 g Na_2CO_3
		Use ~ 400 ml filtered H_2O to dissolve
		Make to 500 ml with filtered H_2O

5.1.2 β-galactosidase assay kit

The commercially available β-galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega, US, CAT number E2000) was used for all β-galactosidase assays.

5.1.3 Composition of yeast media (Esposito and Esposito, 1969; Roth and Halvorson, 1969; Sherman, 1991; Wickerham, 1951)

The composition of media used in this work is outlined below for yeast peptone dextrose (YPD) agar and broth, YPD + G418 agar and yeast peptone acetate (YPAc) agar (Table 3). Pre-sporulation agar 2 – uracil (PSP2 – U) agar, synthetic dextrose (SD) agar, SD – U agar and broth, SD without amino acids (w/o aa) + U + 2 mM L-Leucine (L-Leu) agar and SD

w/o aa + 2 mM L-Leu broth is also outlined in Table 4, with the adjusted concentration of stocks used. PSP2 – U agar required the addition of 50 mM K-phthalate, pH 5.0 (VWR, US), 0.67% (w/v) yeast nitrogen base without amino acids (YNB w/o aa) (Formedium, UK), 0.10% (w/v) bacto yeast extract (Formedium), 1% (w/v) KOAc (Thermo Scientific, US) and 2% (w/v) agar (Formedium). All other media that are included in Table 4 also required the addition of 0.67% (w/v) YNB w/o aa and 2% (w/v) D-glucose (Fisher Chemical, US). 2% (w/v) agar was also added to the media that required it. The supplier and reference/ CAT number of all amino acids are listed in Table 5. An adjusted mass or volume was calculated for each amino acid from its stock, and media was made up to the required volume with filtered H_2O and autoclaved at 121 °C for 20 min before use.

Table 3. Media used in culturing *S. cerevisiae*.

Media	**Composition**	**Concentration (g/l)**
YPD agar	YPD agar powder (Formedium)	70
YPD broth	YPD broth powder (Formedium)	50
YPD + G418 agar	YPD agar powder	70
	G418 (Formedium)	0.4
YPAc agar	Bacto yeast extract	10
	Bacto peptone (Formedium)	20
	KOAc	20
	Agar	20

Table 4. Composition of media with adjusted concentration of amino acids.

Amino acid	**Stock concentration (g/l)**	**Medium with adjusted concentration of amino acids (g/l)**				
		PSP2 - U	SD	SD - U	SD w/o aa + U + 2 mM L-Leu	SD w/o aa + 2 mM L-Leu
L-Tyrosine	0.03	0.03	0.03	0.03	0.00	0.00
Adenine sulphate	1.20	0.02	0.02	0.02	0.00	0.00
L-Arginine-HCl	2.40	0.02	0.02	0.02	0.00	0.00
L-Histidine-HCl	2.40	0.02	0.02	0.02	0.00	0.00
L-Methionine	2.40	0.02	0.02	0.02	0.00	0.00
L-Tryptophan	2.40	0.02	0.02	0.02	0.00	0.00
Uracil	2.40	0.00	0.02	0.00	0.02	0.00
L-Phenylalanine	3.00	0.05	0.05	0.05	0.00	0.00
L-Leucine	3.60	0.10	0.10	0.10	0.10	0.10
L-Isoleucine	3.60	0.03	0.03	0.03	0.00	0.00
L-Lysine-HCl	3.60	0.03	0.03	0.03	0.00	0.00
L-Aspartic acid	4.00	0.10	0.10	0.10	0.00	0.00
L-Glutamic acid	6.00	0.10	0.10	0.10	0.00	0.00
L-Valine	18.00	0.15	0.15	0.15	0.00	0.00
L-Threonine	24.00	0.20	0.20	0.20	0.00	0.00
L-Serine	45.00	0.37	0.37	0.37	0.00	0.00

Table 5. Amino acids used in *S. cerevisiae* culturing media.

Amino acid	**Supplier**	**Reference/ CAT number**
L-Tyrosine	Formedium	DOC0192
Adenine sulphate	Formedium	DOC0229
L-Arginine-HCl	Formedium	DOC0108
L-Histidine-HCl	Formedium	DOC0144
L-Methionine	Formedium	DOC0168
L-Tryptophan	Formedium	DOC0188
Uracil	Formedium	DOC0214
L-Phenylalanine	Formedium	DOC0173
L-Leucine	Formedium	DOC0157
L-Isoleucine	Formedium	DOC0152
L-Lysine-HCl	Formedium	DOC0161
L-Aspartic acid	Formedium	DOC0121
L-Glutamic acid	Calbiochem, USA	3510
L-Valine	Formedium	DOC0197
L-Threonine	Formedium	DOC0185
L-Serine	Formedium	DOC0181

5.1.4 *S. cerevisiae* strains

The organism used in this work was *S. cerevisiae*, with the parental (S288C) BY 4700-064 strain being provided by Dr Martin Schröder (Durham University, UK). *gcn2*Δ and *gcn4*Δ strains were constructed using PCR products from the pFA6kanMX2 plasmid. All strains and their genotypes are outlined in Table 6 below.

Table 6. *S. cerevisiae* strains used in all protein, β-galactosidase and growth assays.

Strain	**Genotype**
BY 4700-064	BY MAT**a** *ura3Δ0*
*gcn2*Δ	BY 4700 *gcn2Δ::kanMX2*
*gcn4*Δ	BY 4700 *gcn4Δ::kanMX2*

5.1.5 *GCN2* and *GCN4* deletion and genotyping primers

All forward and reverse primers used in the isolation of *kanMX2* for the deletion of *GCN2* (6048K, 6049K) and *GCN4* (H9718, H9719) were provided by Dr Martin Schröder. Forward and reverse primers used in genotyping transformants to confirm the deletion of *GCN2* (H9694, H9722) and *GCN4* (H9694, H9720) and to confirm the absence of the WT locus from the *gcn2*Δ transformants (H9723, H9722) were also provided by Dr Martin Schröder. Both forward and reverse primers for the confirmation of the absence of the WT locus from the *gcn4*Δ transformants (H9813, H9814) were designed and ordered externally. The sequence of these primers, their supplier and their melting temperature (Tm) are presented in Table 7.

Table 7. Forward and reverse PCR deletion and genotyping primers.

Primer	**Supplier**	**Sequence**	**Tm (°C)**
Forward primer 6048K	Biomedical Research Core Facilities, DNA Synthesis Core, University of Michigan, US	5′-AGCCTACTTGCGACAACATTCGTGAAACTAGAGGCCTTTGGGCTTTATCACAGCTGAAGCTTCGTACGC-3′	79.0
Reverse primer 6049K	Biomedical Research Core Facilities, DNA Synthesis Core, University of Michigan, US	5′-TATAACATTACATTTTGCGATGACCCCAATGTATCCTTATACCGCTCCAGAGGCCACTAGTGGATCTG-3′	76.3
Forward primer H9718	Eurogentec, Belgium	5′-AGATTAAATTCTTATCTAAGTGAATGTATCTATTTCGTTATACACGAGAAGCTTCGTACGCTGCAGG-3′	64.5
Reverse primer H9719	Eurogentec	5′-TTAAATCATTATTATTACTAAAGTTTTGTTTACCAATTTGTCTGCTCAAGGCCACTAGTGGATCTG-3′	62.7

Forward primer H9694	IDT, US	5′-GATTGCCCGACATTATCGCG-3′	56.6
Reverse primer H9722	IDT	5′-TGTTGACAAAGAGCCGGTTG-3′	55.9
Reverse primer H9720	IDT	5′-CCGTAACGGTTACCTTTCTGTC-3′	55.4
Forward primer H9723	IDT	5′-CCCATTCTGAAGGTGGTTGT-3′	55.1
Forward primer H9813	IDT	5′-AACATTGGAGTTGAATCAGTGC-3′	53.8
Reverse primer H9814	IDT	5′-ACCAATTGCTATCATGTACCCG-3′	55.0

5.1.6 Antibiotic resistance and reporter plasmids

The plasmid maps display the pFA6kanMX2 plasmid, which contains the *kanMX2* gene that confers resistance to G418 (Wach *et al*., 1994) (Fig 2). The centromeric plasmids Z691, p180 and p227 are also displayed, which contain a *URA3* gene and the *KAR2-lacZ*, *GCN4-lacZ* and *GCN4*Δ*4uORF-lacZ* reporters respectively (Mori *et al*., 1993; Hinnebusch, 1985; Mueller and Hinnebusch, 1986) (Fig 3). All plasmids were provided by Dr Martin Schröder.

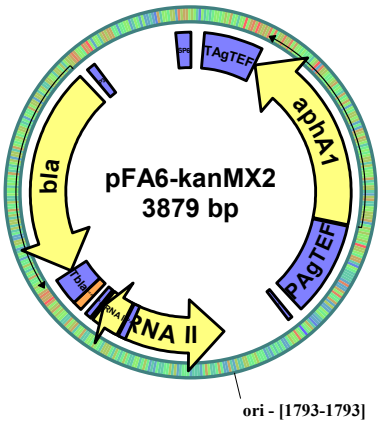

Fig 2. pFA6kanMX2 plasmid used in *GCN2* and *GCN4* gene deletions, and to confer a G418 resistance marker (*kanMX2*) to the deletion strains after excision via PCR.

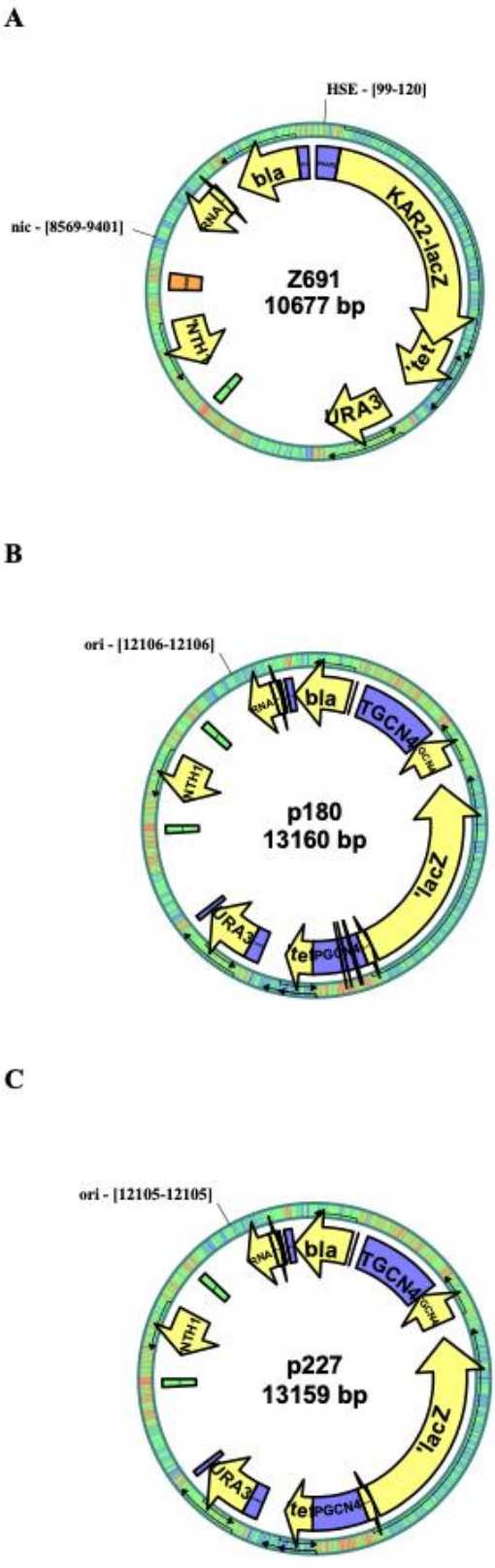

Fig 3. Z691 (**A**), p180 (**B**) and p227 (**C**) reporter plasmids containing the *URA3* gene, used to transform BY 4700-064, *gcn2*Δ and *gcn4*Δ *S. cerevisiae* strains for β-galactosidase/ protein (U/g) quantification.

5.2.1 Production of deletion constructs by PCR (Wach *et al*., 1994)

A master mix was prepared for the isolation of a PCR fragment of *kanMX2* from 25 ng/µl pFA6kanMX2 plasmid with DNA sequence homology corresponding to sequences upstream and downstream of the *GCN2* gene of interest (Table 8), and the *GCN4* gene of interest (Table 9). A negative control of 1 X TE, pH 8.0 was used alongside both PCR reactions. All samples were run for 30 cycles in a G-Storm GS04822 thermocycler for the specified temperatures and times in Table 10 for the *GCN2* and *GCN4* deletion samples. Primers 6048K and 6049K were used for the isolation of a 1442 bp product for the deletion of *GCN2*. For the deletion of *GCN4*, primers H9718 and H9719 were used to produce a 1436 bp product.

Table 8. Master mix for *GCN2* deletion fragment.

Component	**Volume for 1 sample (µl)**
5 X Colourless GoTaq® Flexi buffer (Promega)	20
2 mM dNTPs	10
25 mM $MgCl_2$ (Promega)	8
5 U/µl GoTaq® G2 Flexi DNA polymerase (Promega)	1
100 µM Forward primer 6048K	1
100 µM Reverse primer 6049K	1
25 ng/µl pFA6kanMX2 **OR** 1 X TE, pH 8.0	1
Sterile H_2O	58
Total volume	100

Table 9. Master mix for *GCN4* deletion fragment.

Component	**Volume for 1 sample (µl)**
5 X Colourless GoTaq® Flexi buffer	20
2 mM dNTPs	10
25 mM $MgCl_2$	8
5 U/µl GoTaq® G2 Flexi DNA polymerase	1
100 µM Forward primer H9718	1
100 µM Reverse primer H9719	1
25 ng/µl pFA6kanMX2 **OR** 1 X TE, pH 8.0	1
Sterile H_2O	58
Total volume	100

Table 10. PCR cycle conditions to isolate *kanMX2* for *GCN2* and *GCN4* deletions.

PCR step	**Temperature (°C)**	**Time (s)**
Denaturation	94	120
Annealing	94	30
	51	30
	72	150
Extension	72	600
	10 (*GCN2*), 4 (*GCN4*)	∞

5.2.2 DNA precipitation

To all samples, 10% 3 M NaOAc, pH 6.0 was added. Three volumes of 100% EtOH (stock) were added to each sample and mixed well by inversion. All samples were stored at -20 °C overnight to precipitate. Following precipitation, samples were centrifuged at 14489 g, 7 °C for 30 min and the supernatant discarded. To each sample, 500 µl of 70% EtOH was added and vortexed. Samples were centrifuged again for 15 min, the supernatant discarded and were air dried for 15 min. This step was repeated

with 200 µl of 70% EtOH. 2.5 µl 1 X TE, pH 8.0 buffer was layered on each sample, and all samples were stored overnight at 4 °C. Samples were pooled with 4 µl 1 X TE. The concentration of each pooled sample was read at a 1:200 dilution made with 1 X TE in a 96-well plate (Greiner Bio-One, Austria, CAT number 655801) at 260, 280 and 320 nm on a SpectraMax 190 microplate reader via Warburg-Christian Nucleic Acid Quantitation (Warburg and Christian, 1942).

5.2.3 Yeast growth conditions

S. cerevisiae strains were revived from frozen stock stored at -80 °C, plated on SD agar, and grown for four days at 30 °C. A matchhead of cells on a sterile toothpick were pre-cultured in 3 ml YPD broth in an Infors HT incubator shaker at 250 rpm and 30 °C overnight for strain transformations and one day for isolation of yeast genomic DNA experiments. 20 ml YPD broth was used in all transformation experiments to grow cultures to an OD_{600} of 0.8-1.0. For isolation of yeast genomic DNA, 50 ml YPD was used to grow cultures to an OD_{600} of 6.0-7.0.

Strains transformed with reporter plasmids were grown in 4 ml SD - U broth (balanced medium) or SD w/o aa + 2 mM L-Leu broth (unbalanced medium) for one day in an Infors HT incubator shaker at 250 rpm and 30 °C for UPR and GAAC experiments. Cultures were grown to an OD_{600} of 0.3-0.8 in 50 ml SD - U or SD w/o aa + 2 mM L-Leu broth.

All *S. cerevisiae* strains without reporter plasmids were revived on YPD agar plates and grown at 30 °C for two days for growth assays.

5.2.4 Measurement of cell density by light scattering

The optical density (OD) of pre-cultures and cultures were measured at a 1:20 dilution for transformation experiments and 1:10 for isolation of yeast genomic DNA experiments using YPD at an OD_{600} in a WPA Biowave CO8000 cell density meter. The OD of the pre-cultures and cultures were read at 1:10 at an OD_{600} in SD - U or SD w/o aa + 2 mM L-Leu broth for UPR and GAAC experiments.

5.2.5 Strain and reporter plasmid transformations

Cultures were placed into 50 ml centrifuge tubes and centrifuged at 2250 g at 4 °C for 2 min, and the supernatant discarded. Pellets were put on ice and 9.5 ml 1-step buffer was added. Pellets were resuspended by vortexing and were centrifuged again. The supernatant was discarded, and pellets placed back on ice to allow any remaining 1-step buffer to drain to the bottom of the tubes to be discarded. 90 µl 1-step buffer was added to the pellets for each sample to be transformed, with an additional 90 µl added, to ensure there was enough suspension per sample. Pellets were resuspended by vortexing, and 90 µl of the suspension was added to either a total of 25 µg/µl PCR fragment for the strain transformation or a total of 200 ng/µl for the reporter plasmid transformations in a 1.5 ml tube. 5 µl 1 X TE, pH 8.0 was used as a negative control. 10 µl 10 mg/µl Yeastmaker™ Carrier DNA (Takara, Japan) (denatured via heating to 100 °C for 5 min) was also added

to each sample. All samples were vortexed for 30 s at maximum speed and then heat shocked for 30 min at 42 °C in a water bath. Samples were then centrifuged at room temperature at 17000 g for 15 s, the supernatant discarded, and 200 µl sterile H_2O added. Pellets were resuspended, and the suspensions spread on YPD agar or SD - U agar for reporter plasmid transformations and incubated for one day at 30 °C or until optimal growth was observed.

5.2.6 Replica plating and transformant isolation

Cells were replica plated from YPD agar or SD - U agar for reporter plasmid transformations, using a piece of sterile velvet, which was placed over a replica plater, and the agar plate lightly tapped to transfer colonies. These colonies were transferred to YPD + G418 agar or PSP2 - U agar (reporter plasmids) using the same technique. Plates were incubated at 30 °C until colonies were grown. YPD + G418 plates were replica plated onto YPAc agar, using the same technique and were grown overnight at 30 °C to screen for petite cells. From PSP2 - U plates, colonies were streaked onto SD - U plates, grown for three days at 30 °C, and then streaked onto YPAc plates and grown for three days at 30 °C. Colonies were chosen that matched on the YPD + G418 plate and the YPAc plate to be streaked from each YPAc plate onto YPD agar and grown overnight at 30 °C. A colony from each plate was then streaked onto a YPAc plate and incubated overnight at 30 °C.

5.2.7 Isolation of yeast genomic DNA by chemical lysis of yeast cells (Cryer *et al*., 1975; Philippsen *et al*., 1991; Smith and Halverson, 1968)

Cultures were centrifuged at 2250 g at 4 °C for 2 min in a 50 ml weighed centrifuge tube. The supernatant was discarded and the cells were washed with 10 ml 50 mM EDTA. This was repeated twice. Cells were then washed with 10 ml 0.15 M NaCl, 0.1 M EDTA, 2% (w/v) SDS, pH 10.0 (provided). Samples were weighed, and the weight of the 50 ml tube before the addition of the sample was subtracted to give the wet weight. (This volume was equal to one volume, which would be used later in the protocol). Samples were then stored for 6 h at -20 °C. The cell pellets were thawed and three volumes 0.15 M NaCl, 0.1 M EDTA, 2% (w/v) SDS were added, then 67 µl β-mercaptoethanol was added. All samples were placed into a Hybrid™ Micro-4 rotator at 65 °C overnight. The samples were then transferred into 12 ml tubes placed on ice, 1.5 ml ice-cold KOAc was added to 1.67 M from a 5 M stock (provided), and the samples were mixed by inversion. All samples were incubated for 60 min on ice and then centrifuged at 20000 g at 4 °C for 30 min. The supernatant was transferred to a fresh tube and one volume ice-cold 100% EtOH was overlayed on top. A Fisher Brand 76111 rotary mixer was used to slowly mix the samples, which were then centrifuged again at 12000 g, 4 °C for 10 min. The supernatant was discarded, and the remaining liquid drained out of the tubes by inverting them on tissue for 1 to 2 min. One volume of 50% EtOH was added and all samples were vortexed and then incubated at room temperature for 5 min. The samples were again centrifuged at 12000 g, 4 °C for 10 min and inverted on tissue as previously mentioned. The cell pellets obtained were then air dried at room temperature for 15 min and any liquid drained at the

bottom of each tube discarded. 2.5 µl 20 mg/ml DNase-free RNase in 10 mM NaOAc, pH 7.0 (Sigma, Japan) and 147.5 µl 1 X TE, pH 8.0 were then added to each sample and were incubated overnight at 4 °C. All samples were then transferred to 1.5 ml tubes and were incubated in a 37 °C water bath for 60 min. DNA was extracted twice by adding one volume buffered phenol/$CHCL_3$/isoamylalcohol (25:24:1) (v/v/v), 0.1 M Tris-HCl, pH 8.0 (Fisher Bioreagents, US), and by centrifuging the samples at 12000 g at room temperature for 5 min. In a fresh 1.5 ml tube, the aqueous layer was transferred and one volume $CHCl_3$/isoamylalcohol (24:1) (provided) was added, then centrifuged again. One volume 2-propanol was added to each sample and was slowly mixed. Each sample was centrifuged again at 20000 g at 4 °C for 20 min and the supernatant discarded. The obtained DNA was washed with one volume 50% (v/v) 2-propanol by flicking each tube and incubating for 5 min at room temperature. Centrifugation of the samples was repeated, and the supernatant discarded. The obtained DNA was air dried at room temperature for 15 min and then redissolved overnight at 4 °C in 50 µl 1 X TE.

5.2.8 Burton assay (Burton, 1956; Gendimenico *et al*., 1988; Waterborg and Matthews, 1985)

The concentration of DNA present in each transformant was determined by a Burton assay. In a 1.5 ml tube, each sample was diluted 1:10 with 1 X TE, pH 8.0 and 50 µl 1 N perchloric acid was added. Samples were mixed and incubated at 70 °C for 15 min. 100 µl diphenylamine reagent (prepared on that day) was added. Samples were mixed and incubated at 50 °C for 3 h. 180 µl of each sample was transferred into a well of a 96-well plate,

alongside a blank (250 µl H_2O) and standards (0, 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/ml) made from 400 µg/ml sheared salmon sperm DNA (Invitrogen, US) and 1 X TE. All were read at 595 and 650 nm in a SpectraMax 190 microplate reader.

5.2.9 Genotyping of deletion strains by PCR

A master mix was prepared each for the confirmation of the deletion of *GCN2* and *GCN4* (Table 11), and for the confirmation of the absence of the WT locus from each deletion strain (Table 12). 1 ng/µl *S. cerevisiae* WT strain was used as a positive control and 1 X TE, pH 8.0 was used as a negative control. Samples were run for 30 cycles in a G-Storm GS04822 or GS0001 thermocycler for the specified temperatures and times in Table 13 for the *GCN2* and *GCN4* deletion strains. Primers H9694 and H9722 were used for the confirmation of the deletion of *GCN2* (580 bp product) (1) and H9694 and H9720 for *GCN4* (1) (623 bp). To confirm the absence of the WT locus from the *GCN2* strain (2), primers H9723 and H9722 were used to give a 463 bp product. To confirm this absence in the *GCN4* strain (2), primers H9813 and H9814 were used to produce a 405 bp product.

Table 11. Master mix for *GCN2* and *GCN4* deletion confirmation.

Component	**Volume for 1 sample (µl)**
5 X Green GoTaq® Flexi buffer (Promega)	4.0
2 mM dNTPs	2.0
25 mM $MgCl_2$	1.6
5 U/µl GoTaq® G2 Flexi DNA polymerase	1.0
100 µM Forward primer H9694 (*GCN2*, *GCN4*)	0.2
100 µM Reverse primer H9722 (*GCN2*) H9720 (*GCN4*)	0.2
1 ng/µl sample DNA **OR** 1 ng/µl WT DNA **OR** 1 X TE, pH 8.0	~ 1.0
Sterile H_2O	~ 10.0
Total volume	20.0

Table 12. Master mix for *GCN2* and *GCN4* WT absence confirmation.

Component	**Volume for 1 sample (µl)**
5 X Green GoTaq® Flexi buffer	4.0
2 mM dNTPs	2.0
25 mM $MgCl_2$	1.6
5 U/µl GoTaq® G2 Flexi DNA polymerase	1.0
100 µM Forward primer H9723 (*GCN2*) H9813 (*GCN4*)	0.2
100 µM Reverse primer H9722 (*GCN2*) H9814 (*GCN4*)	0.2
1 ng/µl sample DNA **OR** 1 ng/µl WT DNA **OR** 1 X TE, pH 8.0	~ 1.0
Sterile H_2O	~ 10.0
Total volume	20.0

Table 13. PCR cycle conditions to genotype the deletion of *GCN2* and *GCN4* (1) and the absence of the WT locus (2).

PCR step	**Temperature (°C)**	**Time (s)**			
Denaturation	94	120			
Annealing	94	30			
	55	30			
	72	*GCN2* (1)	*GCN2* (2)	*GCN4* (1)	*GCN4* (2)
		35	28	37	24
Extension	72	600			
	10	∞			

5.2.10 Visualisation and genotyping of deletion strains by agarose gel electrophoresis

To visualise deletion strains to confirm the deletion of both target genes and the absence of the WT locus, agarose gel electrophoresis was carried out. A 0.8% (w/v) agarose gel (agarose powder (Fisher Bioreagents)) was used for strain visualisation and a 1% (w/v) gel was used in genotyping the strains. Agarose was dissolved in a microwave in 1 X TAE. 0.5 µg/ml ethidium bromide (provided) was added once the solution had cooled for 5 min. 12 µl sample was prepared for deletion strain visualisation using sample DNA and 2 µl 6 X TriTrack DNA Loading Dye (Thermo Scientific). Samples were made up to 12 µl with sterile H_2O and were ran alongside 4 µl GeneRuler 1 Kb (Thermo Scientific) at 100 V for 2 h in 1 X TAE buffer in the cold. For genotyping experiments, PCR samples were directly placed into the gel at 20 µl and ran alongside 2 µl GeneRuler 1 Kb at 75 V for 45 min in 1 X TAE. Gel images were captured using an iBright 1500 Imager™.

5.2.11 *S. cerevisiae* culturing for the UPR and GAAC

S. cerevisiae was cultured for UPR experiments, where a 15 ml sample was taken and put into a 15 ml centrifuge tube, and centrifuged at 2250 g, 4 °C for 2 min. The supernatant was discarded, and the pellet stored at -20 °C for future use. To the remaining 35 ml culture, 2 mM DTT was added from a 1 M stock and cultured for 2 h. At 1 and 2 h after the addition of DTT, a 15 ml sample was extracted, centrifuged and frozen as written above for each sample. For experiments concerning the GAAC, samples were cultured, centrifuged and stored as above. However, only one 15 ml sample was extracted from each culture and no drug treatment was used.

5.2.12 Protein extraction for β-galactosidase assays

Samples were thawed at room temperature and put on ice, where 1 ml sterile H_2O was added to each sample. They were then transferred to a 2 ml flat-bottom microcentrifuge tube (Sarstedt, REF number 72.693.005) and centrifuged at 12000 g at room temperature for 1 min. The supernatant was aspirated, then centrifugation and aspiration repeated. 100 µl 1 X RLB (made from 5 X RLB (Promega)) that was ice-cold was added to each sample and were vortexed before adding approximately 150 mg 0.5 mm acid-washed glass beads (Thistle Scientific, UK). The samples were run at 8500 rpm for three, 10 s cycles in a Precellys Evolution Touch instrument, lysing the cells. For 5 min between cycles, the samples were placed on ice. Following cell lysis, 100 µl 1 X RLB (ice-cold) was added to the samples and were vortexed before centrifugation at 12000 g, 4 °C for 2 min. The supernatant was transferred to a 1.5 ml tube, frozen in liquid N_2 and stored at -20 °C.

5.2.13 Protein assay (Lowry *et al*., 1951; Peterson, 1979)

All samples were thawed at room temperature and placed on ice, then were diluted 1:10 with sterile H_2O in a 1.5 ml tube. 5 µl of each sample were transferred to a well of a 96-well plate in duplicate. 5 µl of a buffer control of 1:10 1 X RLB in H_2O, blank of sterile H_2O, DC Protein Assay Reagent A (Bio Rad, US), Reagent B (Bio Rad) and Bovine Serum Albumin (BSA) (Fisher, US) standards of 0, 62.5, 125, 250, 500, 1000 and 2000 µg/ml were also added in duplicate to separate wells. 25 µl Reagent A was added to each well, followed by 200 µl Reagent B. The plate was then incubated at 500 1/min on an MX-M orbital shaker for 15 min at room temperature. The OD of each sample was then read at 750 nm in a SpectraMax 190 microplate reader.

5.2.14 β-galactosidase assay (Miller, 1972; Rose and Botstein, 1983; Rose *et al*., 1990; Schenborn and Goiffon, 1993)

β-galactosidase standards were prepared by diluting the β-galactosidase 1:100 by adding 99 µl 1 X RLB (ice-cold) to 1 µl 1 U/µl β-galactosidase. This dilution was mixed and put on ice, where 10 µl was then added to 990 µl 1 X RLB (ice-cold), mixed again and put back on ice. Standards of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mU/50 µl were prepared in duplicate in the wells of a 96-well plate using the 1:10000 diluted β-galactosidase and 1 X RLB. A 50 µl blank of 1 X RLB was prepared in duplicate and 50 µl 2 X assay buffer (provided) was also prepared in duplicate. Duplicates of samples transformed with the Z691 plasmid pre-DTT treatment were used undiluted (50 µl). Other samples were also made

up to 50 µl with 1 X RLB at different dilutions in duplicate, and this also varied for the repeated dilution depending on if their optical densities were above or below the standard range (Table 14). To each well, 50 µl 2 X assay buffer was added, the plate covered, and then incubated at 37 °C for 30 min. 150 µl 1 M Na_2CO_3 was added to each well after incubation and any bubbles were removed using a scalpel. The OD of all samples was read at 420 nm in a SpectraMax 190 microplate reader.

Table 14. Dilutions of samples used in the β-galactosidase assay.

Sample	**Starting dilution**	**Repeating dilution**
Z691 transformed strain 1 h post-DTT treatment	1:2	1:10
Z691 transformed strain 2 h post-DTT treatment	1:10	1:50
p180 transformed strain	1:10	1:50 (Above range) Undiluted (Below range)
p277 transformed strain	1:10	1:50 (Above range) Undiluted (Below range)

5.2.15 *S. cerevisiae* strains growth assay

Transformants from all strains were streaked in different segments on SD plates (balanced) and grown for two days at 30 °C. For the unbalanced assay, each transformant was streaked on an SD plate and grown for two days at 30 °C. Then they were streaked in different segments of SD + U + 2 mM L-Leu plates and cultured at 30 °C for two days. Images of the plates were captured using an iBright 1500 Imager™.

5.2.16 Statistical analysis

β-galactosidase/ protein (U/g) was calculated for each sample (n=6) for each reporter assay from the protein assay and β-galactosidase assay, and the mean calculated in GraphPad Prism 8.4.3. Most samples were excluded from the analysis if their mean β-galactosidase/ protein (U/g) was too low. The results are presented as the mean ± standard error of the mean (SEM). A Levene test carried out in Microsoft Excel 16.89.1 was used to test for equal variances for each reporter assay, as the ANOVA test assumes homogeneity of variance. If the data had unequal variances, then its logarithm was used and a Levene test carried out on that. For the assays assessing the UPR, a two-way ANOVA was used to assess differences in timepoints and genotype. A one-way ANOVA was used to determine differences in genotype for assays involving the GAAC. A Tukey's multiple comparisons test was used as a test parameter and multiplicity adjusted p values were calculated following the Tukey test. Differences were denoted as significant if $p<0.05$.

6.0 **Results**

6.1 Construction of *GCN2* and *GCN4 S. cerevisiae* deletion strains

To create the *GCN2* and *GCN4* deletion strains required for this work, an S288C *S. cerevisiae* strain was used. The S288C genetic background was used, as it is non-flocculent and is designed to not have a large nutritional requirement (Mortimer and Johnston, 1986). The strain, BY 4700-064 (BY MAT**a** *ura3*Δ0) was prototrophic for all 20 proteinogenic amino acids. Therefore, supplementation of media with amino acids was not required. This could have possibly prevented the activation of the GAAC, and potential starvation of strains deleted for *GCN2* and *GCN4*, as they cannot activate this pathway to synthesise amino acids (Wek *et al*., 1995). The *GCN2* and *GCN4* deletion strains BY 4700 *gcn2*Δ*::kanMX2* and BY 4700 *gcn4*Δ*::kanMX2* were produced from the BY 4700-064 strain by a PCR mediated gene deletion strategy, utilising the plasmid pFA6-kanMX2 to achieve this (Wach *et al*., 1994). Both gene deletions were carried out using PCR, as primers were designed to remove the *kanMX2* gene, allowing genome integration and subsequent deletion of *GCN2* and *GCN4.* The *kanMX2* allele is an antibiotic resistance marker for G418, which was introduced into the pFA6a plasmid via construction with a pAG224 plasmid, containing the Tn*903* transposon (Wach *et al*., 1994). This is important, as the generated fragments of DNA produced from the PCR strategy were used in the subsequent transformation of the WT BY 4700-064 *S. cerevisiae* strain. Ultimately evidence of G418 resistance conferred by the *kanMX2* gene was used in the selection of transformants from plates supplemented with G418, as the transformed *S. cerevisiae* had become resistant to this

drug through exhibiting aminoglycoside 3'-phosphotransferase activity (Jimenez and Davies, 1980).

Colonies were obtained on YPD + G418 agar from the transformation of the BY4700-064 strain to produce *GCN2* and *GCN4* deletion strains. These colonies also grew on media containing acetate (YPAc agar). The genomic DNA (gDNA) of four transformants from two samples each of the deleted *GCN2* and *GCN4* strains was isolated, and confirmation of this gDNA presence in each transformant carried out by agarose gel electrophoresis (Fig 4 A, B). Seven transformants from the *gcn2*Δ strain produced a band on the agarose gel of similar intensity for transformants CGY 001-01, CGY 001-02, CGY 001-03, CGY 001-04, CGY 001-05, CGY 001-06 and CGY 001-07 (Fig 4 A). One transformant CGY 001-08 had a lower intensity presented on the agarose gel, suggesting a lower concentration of DNA present. However, all bands had a similar size of over 10000 bp. Smearing was also seen for all of the bands, showing that the gDNA extracted may have degraded or too much was loaded into the wells. Additionally, faint bands below 250 bp were seen for transformants CGY 001-01, CGY 001-02, CGY 001-04, CGY 001-06, CGY 001-07 and CGY 001-08, with the highest intensity being seen in CGY 001-02. This could be due to RNA contamination of the samples.

Transformants from two samples of the *gcn4*Δ strain are shown in Figure 4 B. Only four transformants (CGY 002-03, CGY 002-06, CGY 002-07 and CGY 002-08) show noticeable bands of gDNA also over 10000 bp. However, faint bands are also visible for transformants CGY 002-01 and

CGY 002-02, showing that a low concentration of gDNA may have been present. Smeared bands are visible for the four transformants with the most intense bands, suggesting again that either too much sample was added to the wells or the gDNA had degraded. Possible RNA contamination may have occurred for the CGY 002-07 transformant, as a faint band was seen in the agarose gel below 250 bp. No other bands were seen for the other transformants.

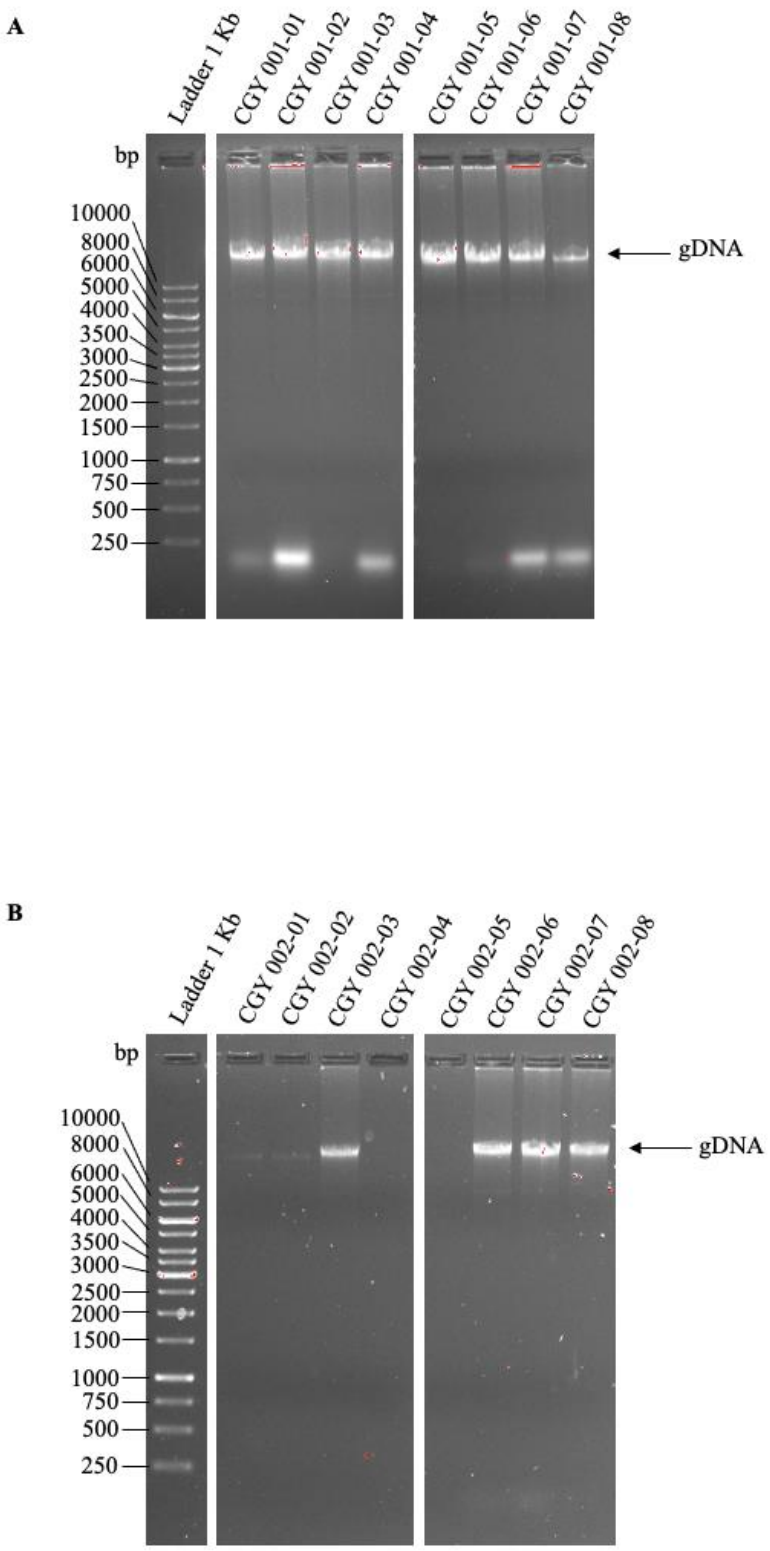

Fig 4. Confirmation via agarose gel electrophoresis of **A** genomic DNA (gDNA) is present in eight transformants from two samples of a *gcn2*Δ strain and **B** showing that in two samples of a *gcn4*Δ strain, at least four transformants contain gDNA.

Both *gcn2*Δ and *gcn4*Δ transformants were then genotyped using agarose gel electrophoresis to confirm the absence of *GCN2* from the *gcn2*Δ transformants (Fig 5 A) and *GCN4* from the *gcn4*Δ transformants (Fig 6 A). Genotyping of the transformants was used to additionally confirm that the WT locus was absent in transformants from both deletion strains (Fig 5 B, Fig 6 B).

All transformants from the *gcn2*Δ strain were confirmed to have *GCN2* deleted, and therefore *kanMX2* integrated, as all transformants produced a band between 500 and 750 bp on the agarose gel (Fig 5 A). No band was present for the positive control because it contained the *GCN2* gene and not *kanMX2* and was therefore not amplified by PCR. Faint bands can be seen under 250 bp for both the positive and negative controls, which as previously stated may indicate possible contamination. Confirmation of the deletion of the WT locus is also shown in all transformants of the *gcn2*Δ strain, as no bands are present (Fig 5 B). A band is however present for the positive control at 500 bp, as it contains *GCN2* and was therefore amplified during PCR. Faint bands below 250 bp are shown for all samples and controls, suggesting possible RNA contamination had occurred.

Transformants isolated from the *gcn4*Δ strain show that only five (CGY-002-02, CGY 002-03, CGY 002-06, CGY 002-07 and CGY002-08) may have *GCN4* deleted and *kanMX2* integrated (Fig 6 A). Bands can be seen between 500 and 750 bp, with a similar intensity being seen for transformants CGY 002-06, CGY 002-07 and CGY 002-08, in comparison to CGY 002-02 and CGY 002-03, which have less intense bands. No band is

present for the positive control, as it contains *GCN4* and not *kanMX2,* so it was not amplified via PCR. Faint bands under 250 bp are seen at increasing intensity from CGY 002-02 to CGY 002-08 and for the positive control, which may indicate RNA contamination. No bands can be seen on the second agarose gel, confirming the absence of the WT locus in the transformants deleted for *GCN4* (Fig 6 B). For the positive control, a band at 500 bp can be seen. This confirms that *GCN4* is not present in all transformants, as only the positive control which contains *GCN4* presents a band. Possible RNA contamination could have occurred in all samples and controls, as bands under 250 bp are present on the agarose gel. This is more probable for CGY 002-01 to CGY 002-03, as the bands under 250 bp were more intense for these samples.

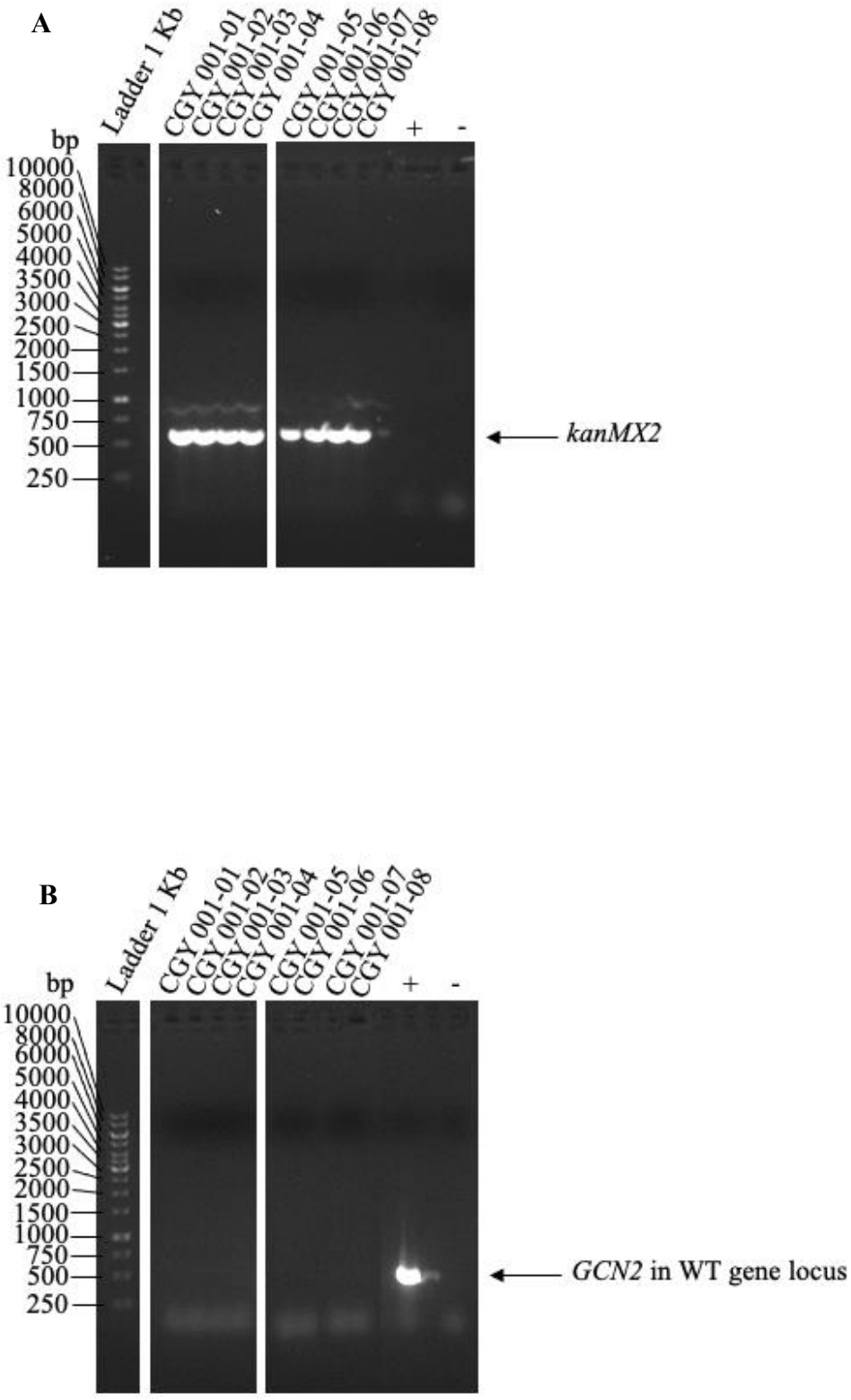

Fig 5. A Integration of *kanMX2* into eight transformants of the *gcn2*Δ strain and **B** the absence of *GCN2* in the eight transformants shown by agarose gel electrophoresis.

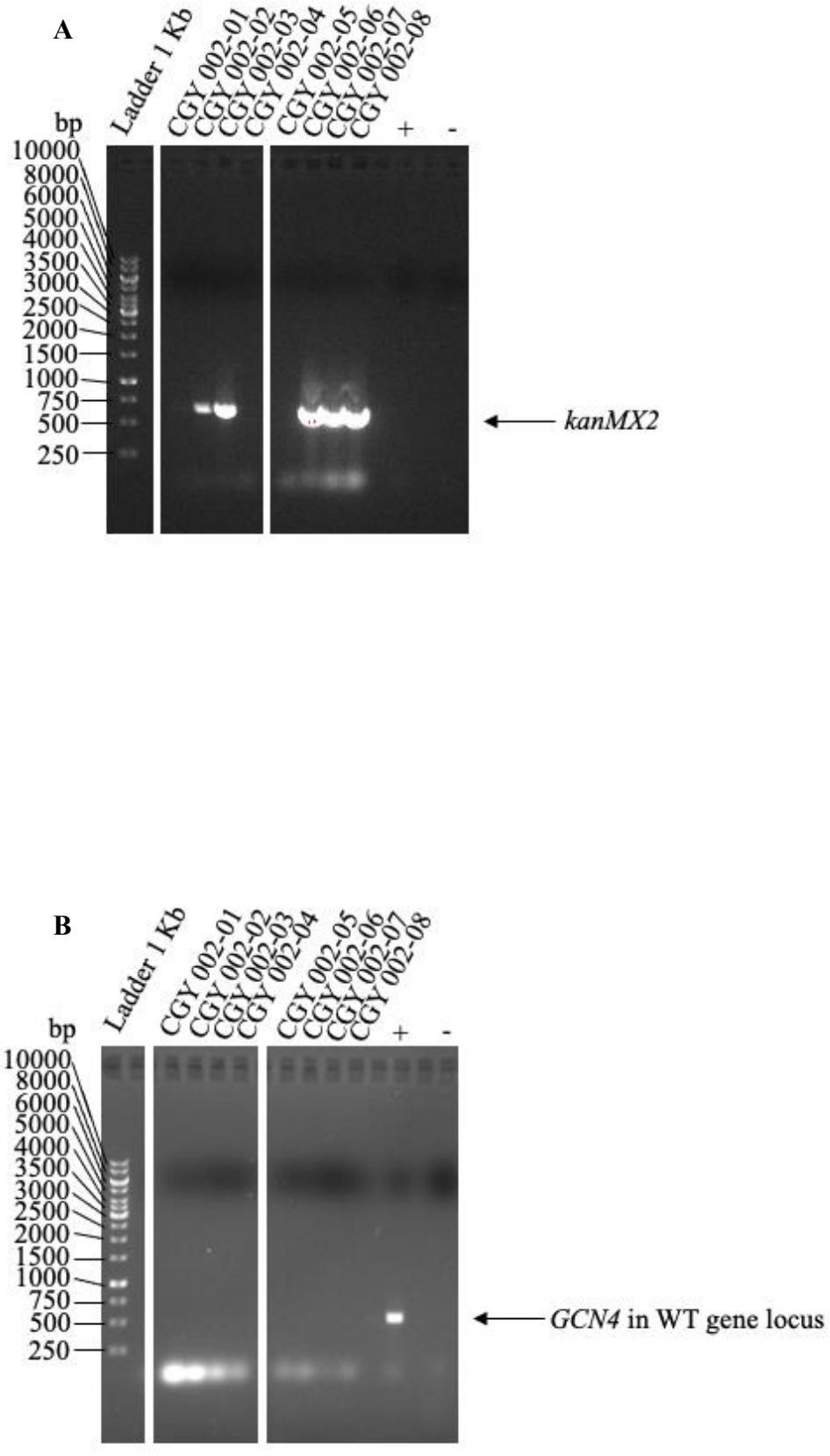

Fig 6. A Integration of *kanMX2* into five possible transformants of the *gcn4*Δ strain and **B** the absence of *GCN4* in transformants shown by agarose gel electrophoresis.

6.2 Gcn4 is required for the UPR under a nutritional deficiency

Following the confirmation of the presence of *kanMX2* and absence of the WT locus in the *gcn2*Δ and *gcn4*Δ *S. cerevisiae* strains, both strains and the WT strain were transformed with the Z691 reporter plasmid. The parental BY 4700-064 (MAT**a** *ura3*Δ*0*) strain (and therefore both deletion strains) were chosen for this investigation, as they are auxotrophic for uracil. Therefore the strains could be transformed with a reporter plasmid, in the case of the UPR investigation, Z691, as this plasmid contains a uracil gene (*URA3* CEN) (Mori *et al*., 1993). Additionally, the Z691 plasmid contains a *KAR2-lacZ* reporter, where the expression of *lacZ* is controlled by the promoter *KAR2* (Mori *et al*., 1992). The *lacZ* gene is derived from *E. coli* and encodes β-galactosidase. When an accumulation of unfolded proteins occurs in the ER, there is a higher expression of *KAR2*, as a result of the UPRE in its promoter region. Therefore, if *KAR2* is expressed, so is *lacZ*, meaning more β-galactosidase activity will occur (Kohno *et al*., 1993). The production of β-galactosidase is important, as it cleaves via hydrolysis o-nitrophenyl-β-d-galactopyranoside (ONPG). ONPG is present in the 2 X assay buffer used in this experiment (refer to Materials and Methods), which has no colour, however when hydrolysed it gives a yellow product, o-nitrophenol, which can be measured by its absorbance at 420 nm (Miller, 1972; Schenborn and Goiffon, 1993). This is relevant to this investigation, as *KAR2* is expressed when Hac1 binds to its UPRE, which means $HAC1^{u}$ mRNA has had to be spliced by Ire1 for this to occur (Nikawa *et al*., 1996; Sidrauski and Walter, 1997). Therefore, in this experiment if the β-galactosidase assay produces a stronger colour change, then it suggests that more *KAR2* gene expression is occurring and that a higher activation of the UPR has also occurred. This would be expected in the parental

S. cerevisiae strain. However, in the *GCN2* and *GCN4* deletion strains, if a lesser colour change is seen during an amino acid imbalance, more so in the *gcn4*Δ strain, then this would suggest that it is needed to induce the *KAR2* gene and is therefore required for the UPR. An imbalance of amino acids was used to elucidate whether Gcn4 is required for the UPR, as Gcn4 is required for the GAAC, which is activated under a nutritional deficiency (Magazinnik *et al*., 2005). Therefore, it can be postulated that under an amino acid balance, no decreasing change in β-galactosidase activity will be seen in the *GCN2* and *GCN4* deletion strains, as the GAAC has not been activated. This result would be expected in the deletion strains under an amino acid imbalance if Gcn4 is not required for the UPR. However as mentioned previously, if it is required, then a decrease in activity would be seen.

β-galactosidase/ protein (U/g) was measured for six samples of independent clones obtained from transformation with the Z691 reporter plasmid each from a WT, *gcn2*Δ and *gcn4*Δ *S. cerevisiae* strain before the addition of DTT in unbalanced medium of SD w/o aa + 2 mM L-Leu. Then 1 h and 2 h after DTT treatment. One sample was omitted from the analysis of the WT and *gcn4*Δ strain before DTT treatment and from the WT strain at 2 h post-DTT treatment due to a low recording of β-galactosidase/ protein (U/g) measured. There were statistical differences observed between all timepoints in the WT strain and in the *gcn2*Δ strain. However, the main result was regarding differences between the strains not the timepoints. It can be seen in Figure 7 that there is a reduction in β-galactosidase/ protein (U/g) in the *gcn2*Δ vs the WT strain at 2 h post-DTT treatment. A reduction

can also be seen at both 1 h and 2 h post-treatment in the *gcn4*Δ vs both the WT and *gcn2*Δ strain. However, statistical differences were only seen at 2 h post-treatment between the *gcn2*Δ strain and the WT strain at a 1.7 ± 0.4-fold decrease ($p<0.0001$). A statistical difference was also seen at this timepoint between the *gcn4*Δ strain and WT strain at a 5.2 ± 1.3-fold decrease ($p<0.0001$) and the *gcn4*Δ strain and *gcn2*Δ strain at a 3.1 ± 0.9-fold decrease ($p<0.0001$). No significant difference was observed at 0 h between strains WT and *gcn2*Δ ($p=0.89$), WT and *gcn4*Δ ($p=0.99$) and *gcn2*Δ and *gcn4*Δ ($p=0.84$). No difference was observed at 1 h between WT and *gcn2*Δ ($p=0.96$), WT and *gcn4*Δ ($p=0.24$) and *gcn2*Δ and *gcn4*Δ ($p=0.15$).

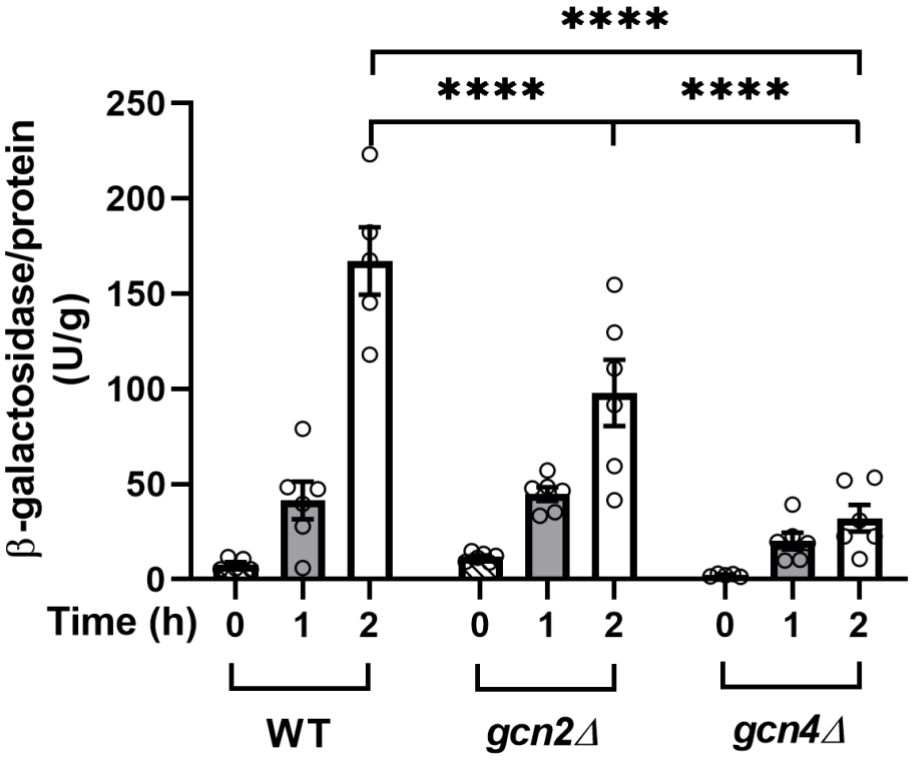

Fig 7. Bar chart represents *β*-galactosidase/ protein (U/g) for samples collected from a wild-type (WT), *gcn2*Δ and *gcn4*Δ strain of *Saccharomyces cerevisiae,* 0, 1 and 2 h post-dithiothreitol (DTT) treatment under an amino acid imbalance. Mean is represented by each bar and error bars represent the standard error of the mean (SEM) for five samples (n=5) for the WT strain at 0 h and 2 h post-DTT treatment, and the *gcn4*Δ strain at 0 h, and six samples (n=6) for all other timepoints. All samples are independent clones obtained from transformation with the *KAR2* promoter-*lacZ* reporter plasmid Z691, and were grown in synthetic defined (SD) medium without amino acids + 2mM L-Leucine. Unfolded protein response (UPR) was elicited using 2mM DTT. Two-way ANOVA was used to assess differences in genotype. Tukey's multiple comparisons test was used to compare means. ****p<0.0001.

6.3 Gcn4 has an established role in the GAAC

To assess the status of the GAAC, in regard to Gcn2 and Gcn4, a β-galactosidase assay was also carried out. For the same reason of the BY 4700-064 parental strain (and deletion strains) being auxotrophic for uracil, a p180 and p227 plasmid was used, as they both contain a *URA3* gene (Hinnebusch, 1985; Mueller and Hinnebusch, 1986). The p180 plasmid (CEN *URA3 GCN4-lacZ*) has a *GCN4-lacZ* reporter, that is a translational fusion gene, which can measure the translation of *GCN4* mRNA (Hinnebusch, 1985). In addition to the p180 plasmid being used in this experiment, a p227 plasmid (CEN *URA3 GCN4*Δ*4uORF-lacZ*) was also transformed into all three *S. cerevisiae* strains, as it also contains a uracil gene. The same reasoning concerning the selection of this reporter for the β-galactosidase assay is used as for the *GCN4-lacZ* reporter. However, this plasmid is constructed so that it has four uORFs of *GCN4* deleted, which should increase the expression of *GCN4*, as the AUG codon ORFs have an inhibitory effect on *GCN4* expression (Mueller and Hinnebusch, 1986). It is expected that the *gcn4*Δ strain will have the highest β-galactosidase activity in comparison to the WT and *gcn2*Δ strains during the *GCN4-lacZ* reporter assay. The WT strain may be able to resolve the imbalance and therefore will show a low β-galactosidase activity. A similar level of activity should be seen in the *gcn2*Δ strain, as this strain will not be able to activate the GAAC without *GCN2* present (Wek *et al*., 1995). The strain with the highest activity should be *gcn4*Δ, as it will not be able to restore the amino acid balance, as it would require *GCN4* to do that. It is also expected that the *GCN4*Δ*4uORF-lacZ* reporter will show more activity than the *GCN4-lacZ* reporter, even under nutritional deficiency, due to the

derepression of *GCN4* from removal of its upstream ORFs (Mueller and Hinnebusch, 1986).

The GAAC response was measured by β-galactosidase/ protein (U/g) levels in a WT, *gcn2*Δ and *gcn4*Δ *S. cerevisiae* deletion strain. Six samples of independent clones obtained from transformation with the p180 reporter plasmid from each strain in unbalanced medium of SD w/o aa + 2 mM L-Leu were used. One sample was omitted from the analysis of all strains due to a varied recording of β-galactosidase/ protein (U/g) measured. The expected result obtained from the assay with the *GCN4-lacZ* reporter shows more activity in the *gcn4*Δ strain, compared to the WT and *gcn2*Δ strain, with the least activity being observed in the *gcn2*Δ strain (Fig 8 A). A significant increase in activity of 23.0 ± 9.5-fold in the *gcn4*Δ strain compared to the WT strain was seen (p<0.05). This increase was also seen between *gcn2*Δ and *gcn4*Δ strains, as the *gcn4*Δ strain showed a 62.9 ± 25.8-fold higher β-galactosidase/ protein (U/g) level (p<0.05). Due to the low amount of activity seen in both WT and the *gcn2*Δ strain, there was no significant difference observed between these strains (p=1.00).

β-galactosidase/ protein (U/g) was also measured from six samples of independent clones obtained from transformation with the p227 reporter plasmid from each strain with the *GCN4*Δ*4uORF-lacZ* reporter in unbalanced medium of SD w/o aa + 2 mM L-Leu. Only five samples were analysed from the WT strain, due to a low recording of β-galactosidase/ protein (U/g). An expected result was also observed from this assay, as the

least activity was seen in the *gcn4*Δ strain compared to both the WT and *gcn2*Δ strain (Fig 8 B). However, only a significant decrease of 4.0 ± 1.5-fold was observed in the *gcn4*Δ strain compared to the WT strain ($p<0.05$). No significant differences were observed between the WT and *gcn2*Δ strain ($p=0.23$) and the *gcn2*Δ and *gcn4*Δ strain ($p=0.30$).

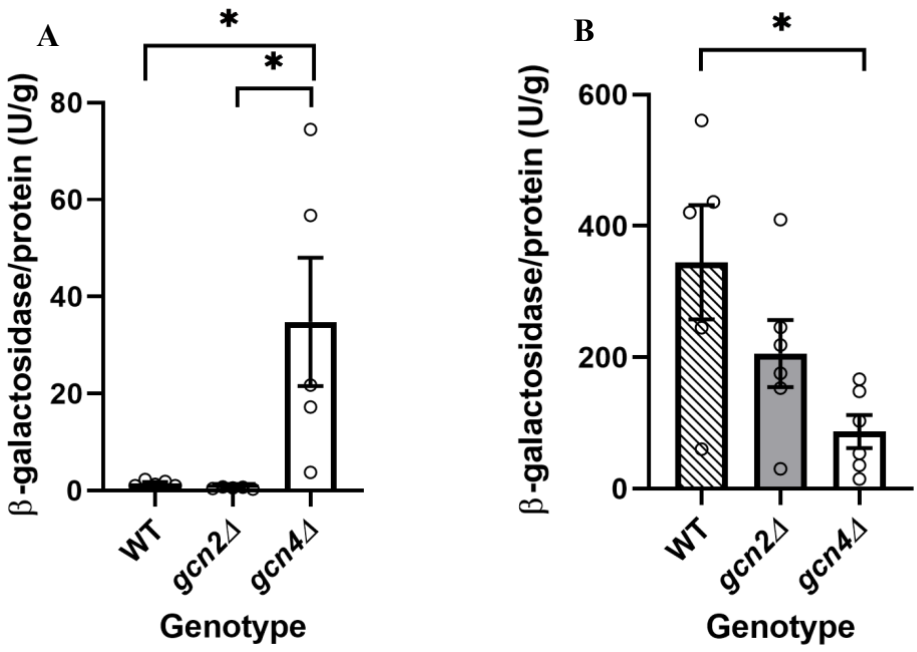

Fig 8. *β*-galactosidase/ protein (U/g) for wild-type (WT), *gcn2*Δ and *gcn4*Δ strains of *Saccharomyces cerevisiae* samples measuring (A) *GCN4*-5'-*lacZ* reporter (p180) and (B) mutant *GCN4*-5'UTR-*lacZ* reporter (p227) under an amino acid imbalance. Mean is represented by each bar and error bars represent the standard error of the mean (SEM). Five samples (*n*=5) were analysed for all strains with the *GCN4-lacZ* reporter (p180). Six samples (*n*=6) were analysed for *gcn2*Δ and *gcn4*Δ strains and five samples (*n*=5) were analysed for the WT strain with the *GCN4*Δ*4uORF-lacZ* reporter (p227). All samples are independent clones obtained from transformation with the p180 or p227 reporter plasmids and were grown in synthetic defined (SD) medium without amino acids + 2mM L-Leucine. One-way ANOVA was used to assess differences in genotype. Tukey's multiple comparisons test was used to compare means. *$p<0.05$.

6.4 Confirmation of a Gcn4 requirement in the UPR and its established role in the GAAC

Following on from the UPR and GAAC investigations under an amino acid imbalance, which was used to activate Gcn4 and therefore elicit the GAAC (Magazinnik *et al*., 2005), the same experimental procedures were used to measure β-galactosidase/ protein (U/g) in WT, *gcn2*Δ and *gcn4*Δ strains. These strains were transformed with Z691, p180 and p227 plasmids, and a medium with a balanced provision of amino acids was used in these assays. To achieve this balanced medium (SD - U), amino acids listed in Table 4 were separately added, with only uracil omitted. The same rationale regarding the use of reporters from each plasmid (Z691 (*KAR2-lacZ*), p180 (*GCN4-lacZ*) and p227 (*GCN4*Δ*4uORF-lacZ*)) is applied to this balanced assay (Hinnebusch, 1985; Mori *et al*., 1993; Mueller and Hinnebusch, 1986). A balanced nutritional supply was used in this assay, which would confirm the previous results because more activity should be seen in the deletion strains in the balanced assay compared to the assays performed in unbalanced media. The reason behind this is Gcn4 is regulated by four ORFs that are upstream of the mRNA of *GCN4* (Mueller and Hinnebusch, 1986). So, if *GCN4* is repressed by these ORFs and an amino acid balance is maintained then the WT, *gcn2*Δ and *gcn4*Δ strains should show similar activity for all reporter assays, as both Gcn2 and Gcn4 are not required to be activated.

Six samples of independent clones obtained from transformation with the Z691 reporter plasmid taken from WT, *gcn2*Δ and *gcn4*Δ *S. cerevisiae* strains were treated with DTT to elicit the UPR in balanced medium of

SD - U. β-galactosidase/ protein (U/g) was measured for each sample before DTT treatment, then 1 h and 2 h post-treatment. Between all timepoints in both the *gcn2*Δ and *gcn4*Δ strain, statistical differences were seen. This was also true between 0 and 2 h, and 1 and 2 h in the WT strain, but genotype differences were the main result sought. A significantly higher level of β-galactosidase/ protein (U/g) was seen in the *gcn2*Δ strain compared to the WT strain at 2 h post-DTT treatment at 1.6 ± 0.3-fold (p<0.01) (Fig 9). This result was also observed at this timepoint for the *gcn4*Δ strain compared to the WT strain at 1.5 ± 0.4-fold (p<0.05). Similar levels of β-galactosidase/ protein (U/g) was recorded pre-DTT treatment for the WT vs *gcn2*Δ strain (p=0.99), WT vs *gcn4*Δ strain (p=1.00) and *gcn2*Δ vs *gcn4*Δ strain (p=0.99). This was also observed 1 h post-DTT treatment for the WT vs *gcn2*Δ strain (p=0.39), WT vs *gcn4*Δ strain (p=0.27) and *gcn2*Δ vs *gcn4*Δ strain (p=0.97). For 2 h post-treatment, similar levels of β-galactosidase/ protein (U/g) was also seen for the *gcn2*Δ vs *gcn4*Δ strain (p=0.98).

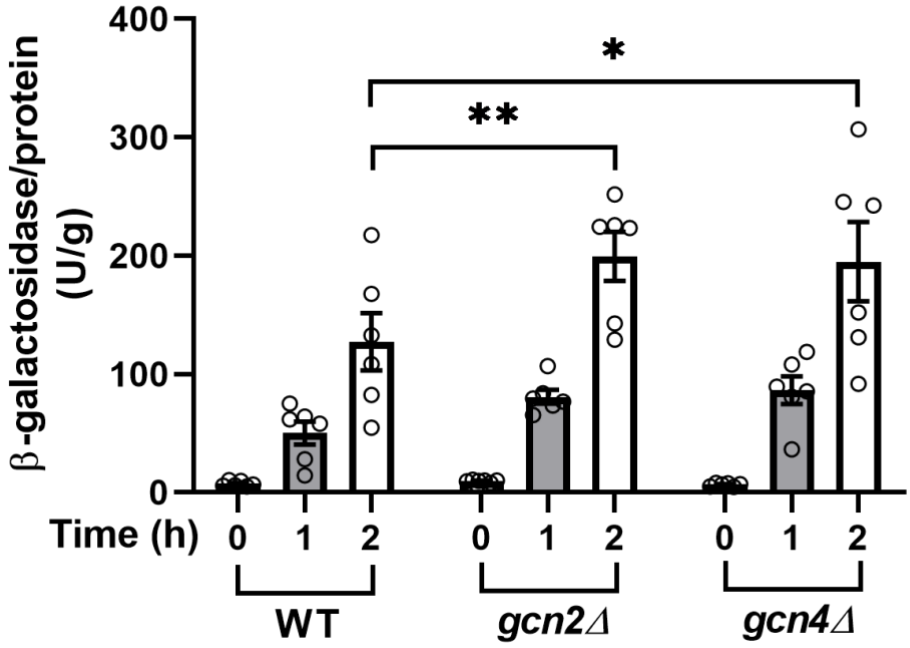

Fig 9. Bar chart showing *β*-galactosidase/ protein (U/g) for samples collected from a wild-type (WT), *gcn2*Δ and *gcn4*Δ strain of *Saccharomyces cerevisiae,* 0, 1 and 2 h post-dithiothreitol (DTT) treatment under an amino acid balance. Mean is represented by each bar and error bars represent the standard error of the mean (SEM) for six samples (*n*=6) of independent clones obtained from transformation with the Z691 reporter plasmid for WT, *gcn2*Δ and *gcn4*Δ strains grown in synthetic defined (SD) medium without uracil. Unfolded protein response (UPR) was elicited using 2mM DTT. All samples were measured using a *KAR2* promoter-*lacZ* reporter (Z691). Two-way ANOVA was used to assess differences in genotype. Tukey's multiple comparisons test was used to compare means. *p<0.05, **p<0.01.

An expected result was again obtained for the assay with the *GCN4-lacZ* reporter. β-galactosidase/ protein (U/g) was measured in six samples of independent clones obtained from transformation with the p180 reporter plasmid each from a WT, *gcn2*Δ and *gcn4*Δ *S. cerevisiae* strain, grown in balanced medium of SD - U. There was very little activity in all strains, with the least activity being seen in the *gcn2*Δ strain (Fig 10 A). The most activity was seen in the *gcn4*Δ strain. Comparisons between strains were not significant, as all strains exhibited similar activity. WT vs *gcn2*Δ (p=0.68), WT vs *gcn4*Δ (p=0.88) and *gcn2*Δ vs *gcn4*Δ (p=0.40).

A higher level of β-galactosidase/ protein (U/g) was measured from the assay using the *GCN4*Δ*4uORF-lacZ* reporter in six samples of independent clones obtained from transformation with the p227 reporter plasmid, each from the *gcn2*Δ and *gcn4*Δ strains, and five samples from the WT strain also grown in balanced medium of SD - U. Five samples were used for analysis of the WT strain due to a low recording of β-galactosidase/ protein (U/g). The highest level of β-galactosidase/ protein (U/g) was recorded in the *gcn2*Δ strain compared to the WT and *gcn4*Δ strain (Fig 10 B). The lowest activity was seen in the WT strain. Similar activity was however seen in all strains, as no significant differences were seen in the WT and *gcn2*Δ strain (p=0.42), the WT and *gcn4*Δ strain (p=0.48) and the *gcn2*Δ and *gcn4*Δ strain (p=0.99).

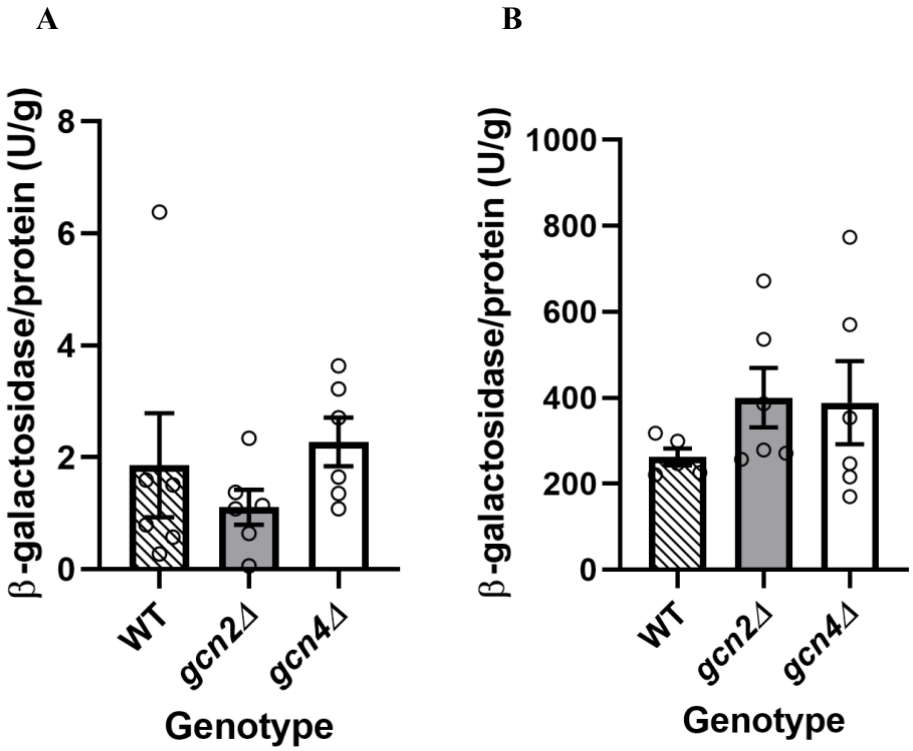

Fig 10. Wild-type (WT), *gcn2*Δ and *gcn4*Δ strain samples of *Saccharomyces cerevisiae* measuring (A) *GCN4*-5'-*lacZ* reporter (p180) and (B) mutant *GCN4*-5'UTR-*lacZ* reporter (p227) under an amino acid balance exhibiting *β*-galactosidase/ protein (U/g). Mean is represented by each bar and error bars represent the standard error of the mean (SEM). Six samples (*n*=6) were analysed for all strains with the *GCN4-lacZ* reporter (p180). Six samples (*n*=6) were analysed for *gcn2*Δ and *gcn4*Δ strains and five samples (*n*=5) were analysed for the WT strain with the *GCN4*Δ*4uORF-lacZ* reporter (p227). All samples are independent clones obtained from transformation with the p180 or p227 reporter plasmids and were grown in synthetic defined (SD) medium without uracil. One-way ANOVA was used to assess differences in genotype. Tukey's multiple comparisons test was used to compare means.

6.5 Correction of *KAR2-lacZ* reporter expression

Differences observed from the results of the reporter assays investigating the UPR could have been independently influenced by the translation rates of the three *S. cerevisiae* strains. This means that differences may have been due to this factor, and not due to DTT treatment to trigger the UPR. The *GCN4Δ4uORF-lacZ* reporter present in the p227 plasmid does not have the four uORFs of *GCN4*, meaning that there is no inhibitory effect on translation of the *GCN4* mRNA. This reporter therefore measures translational activity, even under an amino acid balance (Mueller and Hinnebusch, 1986). When different amino acids are supplemented, such as leucine, all three strains will exhibit different translation rates. The WT strain will have the highest activity, as *GCN2* and *GCN4* are present in this strain. Therefore, when an imbalance of amino acids is created from not supplementing leucine with isoleucine and valine, the WT strain can activate the GAAC to restore the balance of amino acids (KEGG, 2021; Wek *et al*., 1995). However, in the *gcn2Δ* and *gcn4Δ* strains, this may not be possible, as both are required for the GAAC (Hope and Struhl, 1985; Wek *et al*., 1995). This means different translation rates will be recorded by the *GCN4Δ4uORF-lacZ* reporter. These results can be used to correct for the differences in the UPR investigation. To account for differences in translation rates, the mean β-galactosidase/ protein (U/g) was calculated for each strain in the Z691 and p227 reporter assay. The ratio of these means between assays involving the Z691 and p227 plasmid (Z691:p227) was then calculated and analysed.

The mean β-galactosidase/ protein ratio was calculated from six samples of independent clones obtained from transformation with the Z691 and p227 reporter plasmids for WT, *gcn2*Δ and *gcn4*Δ *S. cerevisiae* strains grown in unbalanced medium of SD w/o aa + 2 mM L-Leu (Fig 11 A) and balanced medium of SD - U (Fig 11 B). The WT strain at 0 h and 2 h post-DTT treatment had four samples analysed, due to low β-galactosidase/ protein (U/g) levels. Five samples were included in the analysis for the WT strain at 1 h post-DTT treatment and the *gcn4*Δ strain at 0 h (Fig 11 A). For the WT strain at 0 h, 1 h and 2 h post-DTT treatment, five samples were included in the analysis (Fig 11 B). All strains exhibited similar activity in both unbalanced assay (Fig 11 A) and balanced assay (Fig 11 B). A significant difference in the timepoint of 0 vs 2 h post-DTT treatment was seen for the WT strain. There were no significant differences calculated before DTT treatment between the WT vs *gcn2*Δ strain (p=0.98), WT vs *gcn4*Δ strain (p=1.00) and *gcn2*Δ vs *gcn4*Δ strain (p=0.98). No significant differences were calculated after DTT treatment at 1 h for WT vs *gcn2*Δ strain (p=0.88), WT vs *gcn4*Δ strain (p=0.58) and *gcn2*Δ vs *gcn4*Δ strain (p=0.85), or at 2 h for WT vs *gcn2*Δ strain (p=0.68), WT vs *gcn4*Δ strain (p=0.69) and *gcn2*Δ vs *gcn4*Δ strain (p=1.00) (Fig 11 A).

Significant differences in timepoints were observed for 0 vs 2 h and 1 vs 2 h post-DTT treatment for all strains. However, no differences in genotypes were found for Figure 11 B, as no significant differences were observed before DTT treatment between the WT vs *gcn2*Δ strain (p=1.00), WT vs *gcn4*Δ strain (p=1.00) and *gcn2*Δ vs *gcn4*Δ strain (p=1.00). No significant differences after DTT treatment were reported at 1 h for WT vs *gcn2*Δ strain

(p=1.00), WT vs *gcn4*Δ strain (p=0.80) and *gcn2*Δ vs *gcn4*Δ strain (p=0.83), or at 2 h for WT vs *gcn2*Δ strain (p=0.91), WT vs *gcn4*Δ strain (p=0.56) and *gcn2*Δ vs *gcn4*Δ strain (p=0.80).

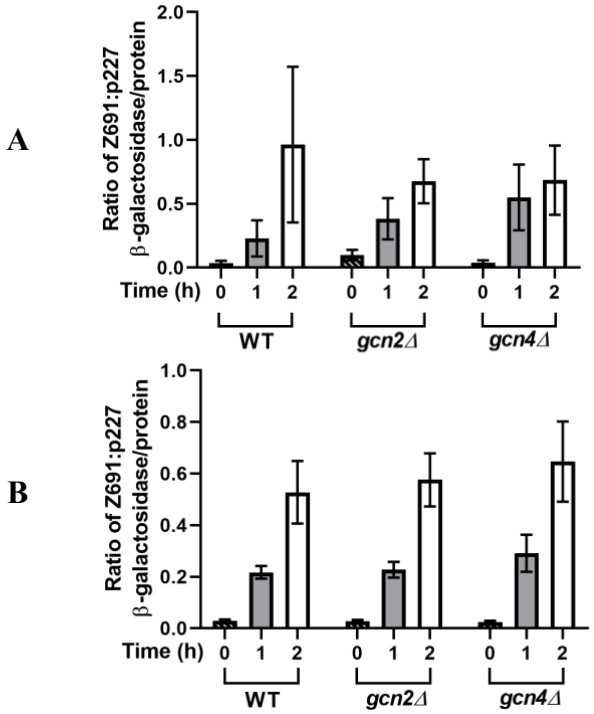

Fig 11. Mean Z691:p227 ratio of *KAR2* promoter-*lacZ* (Z691)/ mutant *GCN4*-5'-*lacZ* (p227) reporters expression showing *β*-galactosidase/ protein for samples collected from a wild-type (WT), *gcn2*Δ and *gcn4*Δ strain of *Saccharomyces cerevisiae,* 0, 1 and 2 h post-dithiothreitol (DTT) treatment under an amino acid imbalance (A) and amino acid balance (B). Mean is represented by each bar and error bars represent the standard error of the mean (SEM) for four samples (*n*=4) for the WT strain at 0 h and 2 h post-DTT treatment, five samples (*n*=5) for the WT strain at 1 h post-DTT treatment and the *gcn4*Δ strain at 0 h. Six samples (*n*=6) were analysed for all other timepoints in Figure A. Five samples (*n*=5) were included for the WT strain at 0 h, 1 h and 2 h post-DTT treatment. Six samples (*n*=6) were analysed for all other strains and timepoints in Figure B. All samples were grown in synthetic defined (SD) medium without amino acids + 2mM L-leucine (A) or SD medium without uracil (B). Unfolded protein response (UPR) was elicited using 2mM DTT. All data are independent clones obtained from transformation with Z691/p227 reporter plasmids. Two-way ANOVA was used to assess differences in genotype. Tukey's multiple comparisons test was used to compare means.

6.6 Growth assay of the WT, *gcn2*Δ and *gcn4*Δ *S. cerevisiae* strains

Following the reporter assays, the growth of the WT (BY 4700-064), *gcn2*Δ and *gcn4*Δ strains that were not transformed with the Z691, p180 and p227 plasmids were carried out, due to slow growth and varying β-galactosidase/ protein (U/g) levels seen in some samples from the same strain. This assay was also performed under balanced (SD) and unbalanced (SD + U + 2mM L-Leu) conditions on agar plates for four pFA6-kanMX2 transformants taken from the *gcn2*Δ and *gcn4*Δ strains, compared to the WT strain. The unbalanced media required uracil supplementation, as all strains were auxotrophic for uracil.

This assay could help to visualise the slow growth of *gcn2*Δ and *gcn4*Δ strains that had a slower growth rate compared to the WT strain when grown under an imbalance of amino acids.

Equal growth can be observed from the first plate on balanced medium for the WT replicates and for transformants 1 and 2 for both the *gcn2*Δ and *gcn4*Δ strains (Fig 12 A). However, on the second plate, less growth can be seen for one of the WT replicates, and for transformant 4 from *gcn2*Δ compared to transformant 3. Less growth is also visible for transformant 3 from the *gcn4*Δ strain compared to transformant 4 of the same strain.

For the WT replicates on the unbalanced medium, equal growth can be seen again on the first plate (Fig 12 B). This is also true for transformants 1 and 2 from the *gcn2*Δ strain, but not for the *gcn4*Δ strain, as less growth is seen in

transformant 2 compared to transformant 1. On the second plate for the unbalanced medium, the WT replicates have both grown equally, as is the same for transformant 3 and 4 from the *gcn2*Δ strain. Less growth is however seen in transformant 3 compared to transformant 4 of the *gcn4*Δ strain.

A

B

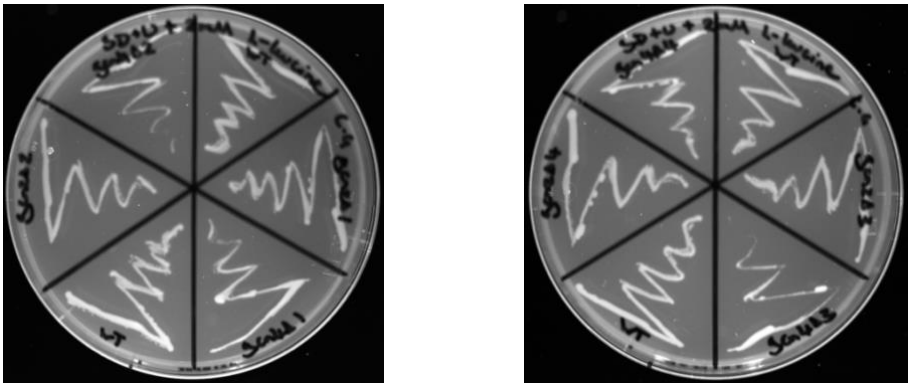

Fig 12. Growth assay confirming growth of two wild type (WT) (BY 4700-064) replicates and four *gcn2*Δ and *gcn4*Δ pFA6-kanMX2 transformants on **A** balanced medium (synthetic dextrose (SD)) and **B** unbalanced medium (SD + uracil (U) + 2mM L-Leucine).

7.0 **Discussion**

The bZIP transcription factor Hac1 has an established preference of binding as homodimers in *S. cerevisiae* (Deppmann *et al*., 2006). However, this transcription factor has been suggested to interact with Gcn4 as a heterodimer to induce UPR targeted genes (Patil *et al*., 2004). Alternatively, it can also be interpreted from the Patil study that Gcn4 is activated upon an imbalance of amino acids and therefore has an indirect role in the UPR, meaning it does not bind to UPREs with Hac1. The aim of this present study was to gather data to distinguish between these two possibilities, and some of the results gathered in this study do support the latter suggestion that Gcn4 has an indirect role in the UPR. My results also suggest that Gcn4 may not be required for the UPR at all, because of reporter assay data for the translation rates of the WT, *gcn2*Δ and *gcn4*Δ strains resulted in no difference being observed in all reporter assays.

7.1 Requirement of Gcn4 to restore an amino acid balance

The results of this study suggest that Gcn4 is indirectly required for the UPR through its role in activating the GAAC. This was hypothesised because under an amino acid imbalance, there is a depletion of amino acids which are used to synthesise proteins. Therefore, activation of the UPR should not occur without Gcn4, as it requires this transcription factor to induce amino acid biosynthetic genes to restore the balance in amino acids used in the production of proteins (Hope and Struhl, 1985) (Fig 13). It is this protein production this is ultimately needed to induce the UPR (Schröder, unpublished), as that is activated by an accumulation of unfolded proteins

(Okamura *et al*., 2000). The results obtained from the reporter assays investigating the UPR and GAAC under an imbalance of amino acids support this idea. Both *GCN2* and *GCN4* deletion strains exhibited a lower level of activity during the UPR compared to the WT strain at 2 h (Fig 7). This result suggests that Gcn2 is required to activate Gcn4, but is not essential in doing so, as the *gcn2*Δ strain had more activity than the *gcn4*Δ strain. This suggests that Gcn4 may have been activated by another general control nonderepressible protein, such as Gcn3. This protein increases the expression of Gcn4 under conditions of starvation, which may describe how the *gcn2*Δ strain had more activity than the *gcn4*Δ strain in this investigation (Hannig and Hinnebusch, 1988). The least activity was seen in the *gcn4*Δ strain, which suggests that Gcn4 is indirectly required during the UPR to restore a balance of amino acids via the GAAC, which could not be fully elicited without *GCN4* in this deletion strain (Hope and Struhl, 1985).

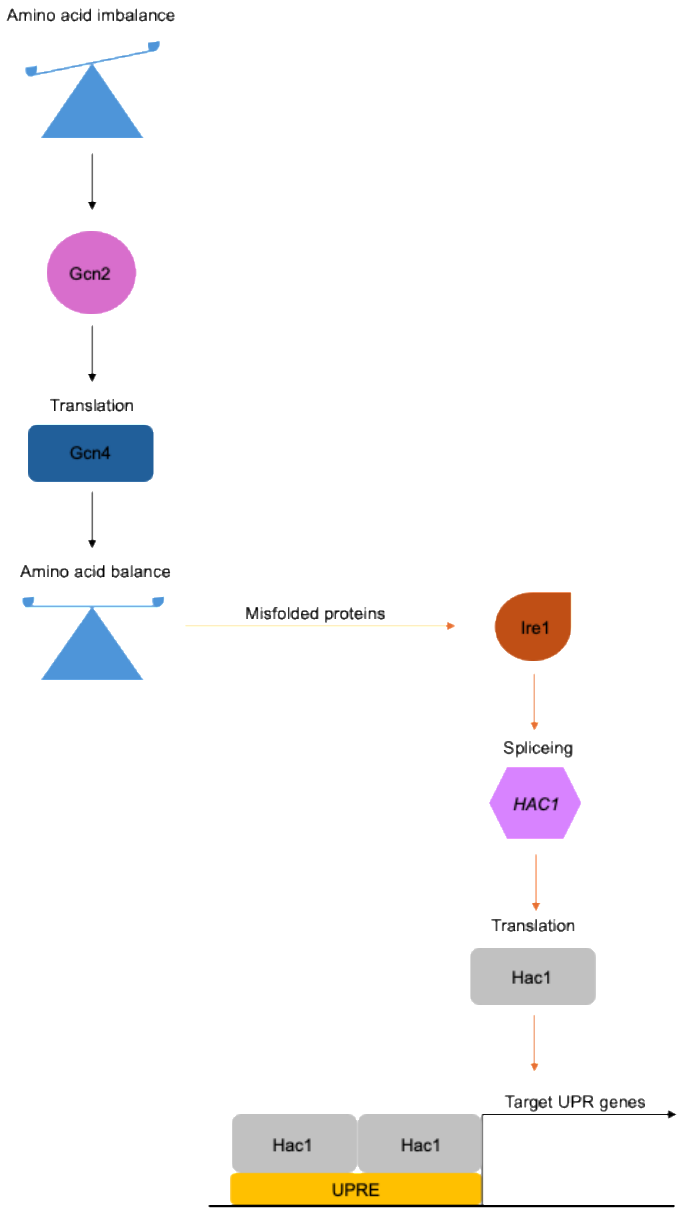

Fig 13. The established separate mechanisms of the general amino acid control (GAAC) (black arrows) and the unfolded protein response (UPR) (orange arrows) in *Saccharomyces cerevisiae* recapitulated from the results of this study. Upon an amino acid imbalance, general control nonderepressible 2 (Gcn2) activates general control nonderepressible 4 (Gcn4) to restore an amino acid balance. However, the amino acids produced are also alternatively suggested to be used in protein production, activating the UPR from an accumulation of unfolded proteins. This activates inositol-requiring 1 (Ire1), that splices the mRNA of *HAC1*, resulting in the translation of Hac1. Two monomers of Hac1 bind to unfolded protein response elements (UPREs) as a homodimer to activate target UPR genes.

To further establish the role of Gcn4 in the GAAC, reporter assays investigating this pathway were carried out with *GCN4-lacZ* reporter on plasmid p180, in which translation of the *lacZ* ORF is under control of four short uORFs (Hinnebusch, 1985). Under an imbalance of amino acids, the WT and *gcn2*Δ strain exhibited very little β-galactosidase/ protein (U/g) activity (Fig 8 A). This suggests that the WT strain had stopped activating the GAAC, as the balance in amino acids had been restored. In the *gcn2*Δ strain, the GAAC had not been activated, as it had required Gcn2 in this instance, which this strain does not possess. The strain with the most activity during this assay was the *gcn4*Δ strain, which suggests that it had not resolved the amino acid balance. This result is suggested from the data, as this strain has Gcn2, which may describe why activity was seen in this strain even when Gcn4 was not present, as Gcn4 is not required to activate translation of the mRNA from the reporter (Hinnebusch, 1985).

The final result obtained from the unbalanced reporter assays using the *GCN4-lacZ* reporter in which the four uORFs in the *GCN4* leader sequence have been mutated, revealed the different translation rates of the WT, *gcn2*Δ and *gcn4*Δ strains, as no uORFs were present to repress translation of the reporter used in this assay (Fig 8 B) (Mueller and Hinnebusch, 1986). The WT strain had the most β-galactosidase/ protein (U/g) activity, compared to the *gcn2*Δ and *gcn4*Δ strains, where *gcn4*Δ exhibited the least activity. This would be expected, as the provision of amino acids included only supplementation with leucine, which created an imbalance (KEGG, 2021). Therefore, the WT strain had a higher level of translational activity, as it contained both *GCN2* and *GCN4* and could therefore translate Gcn4 upon

activation of the GAAC (Hope and Struhl, 1985). This rate of translation was reduced in the *gcn2*Δ strain, but not to the extent of the *gcn4*Δ strain. As previously stated, this may be due to other factors affecting the translation of Gcn4, such as Gcn3 (Hannig and Hinnebusch, 1988). However, in the *gcn4*Δ strain, Gcn4 is required during the GAAC (Hope and Struhl, 1985). This could explain why the least translation occurred in this strain, as *GCN4* was not present.

The reporter assays carried out under a balance of amino acids further support the unbalanced reporter assay data in suggesting that Gcn4 is required indirectly during the UPR (Fig 9). This can be taken from the UPR reporter assay data, as the UPR had been elicited in all strains, as they exhibited similar activity after UPR induction at 1 h. The WT strain exhibited similar activity to the unbalanced reporter assay, which further suggests that the GAAC was elicited in the unbalanced assay in this strain. Both deletion strains show similar activity in this assay at 2 h, that was higher than the WT strain. This shows that Gcn2 and Gcn4 were not required to be activated, as the GAAC was not elicited, meaning all strains could elicit the UPR. Both assays using the *GCN4-lacZ* reporter (Fig 10 A) and the *GCN4*Δ*4uORF-lacZ* reporter (Fig 10 B) under an amino acid balance showed no difference in activity between the three *S. cerevisiae* strains in each assay. This further supports the previous results utilising these reporters, that Gcn4 is required during the GAAC because only under a nutritional deficiency where both Gcn2 and Gcn4 were required, a difference in activity was observed.

7.2 Gcn4 does not have an indirect role in the UPR

To account for the differences in translation rates of the WT, *gcn2*Δ and *gcn4*Δ strains, further analysis was carried out via calculating the mean *β*-galactosidase/ protein ratio between the *KAR2-lacZ* and *GCN4*Δ*4uORF-lacZ* reporters from the Z691 and p227 reporter plasmids (Mori *et al*., 1993; Mueller and Hinnebusch, 1986), to account for translational differences during the UPR reporter assays under an amino acid imbalance and balance. This analysis has revealed no significant differences were present between the WT strain and deletion strains under an imbalance of amino acids (Fig 11 A). The reporter assay carried out under an amino acid balance (Fig 11 B) also shows this. There is a marginal difference in the mean activity between this assay and the unbalanced assay, suggesting Gcn2 and Gcn4 were not required to activate the UPR. Therefore, it can be concluded that the difference observed prior to correction for the translation rates, was from limiting amino acids. When leucine was supplemented into the media, without isoleucine and valine, an imbalance was created, as biosynthesis of these amino acids share several steps. This would have activated the GAAC to try to restore the balance using Gcn2 and Gcn4 in this pathway (Fig 13), which explains the results obtained for the *GCN2* and *GCN4* deletion strains in the reporter assays (KEGG, 2021; Wek *et al*., 1995).

The growth assay performed with all strains before reporter plasmid transformation also supports this conclusion, as very little differences in strain growth were observed on both unbalanced and balanced media (Fig 12). Singular colonies were not obtained in this assay, so it cannot be

definitively concluded that less growth occurred from one strain to the other. However, less growth was initially expected from both the *gcn2*Δ and *gcn4*Δ strains under amino acid imbalance, which was not seen. Although the result of this assay is not conclusive, it can help to visualise the slow growth which was observed in all strains in unbalanced medium, due to the strains trying to synthesise various amino acids to restore the nutritional balance (Hope and Struhl, 1985).

This conclusion of Gcn4 not having an indirect role in the UPR differs from the conclusions of the Patil study, which suggested that Gcn4 has a role in the UPR either directly by binding as a heterodimer with Hac1, or indirectly by correcting an imbalance of amino acids. Both my study and the Patil study used drug treatment to induce the UPR, however, DTT was used in this study and tunicamycin (Tm) was used in the Patil study to both show the interaction between Hac1 and Gcn4 and to inadvertently show that Gcn4 is required for the GAAC. Therefore, this could account for differences in the results of this study in comparison to the Patil study, as DTT elicits the UPR by inhibiting the formation of disulphide bonds and Tm elicits the UPR by the alternative mechanism of inhibiting N-linked glycosylation (Braakman *et al*., 1992; Hauptmann *et al*., 2006). Later studies also used Tm to induce the UPR to produce findings supporting the Patil study, in which Gcn4 and Hac1 indirectly affect each other and that they may also regulate target gene transcription by binding to promoter half binding sites (Herzog *et al*., 2013). However, this more recent study also created conditions similar to Patil *et al,* as it used an *S. cerevisiae* strain that was auxotrophic for uracil and tryptophan, then transformed it with reporter plasmids that

contained a uracil gene. Therefore, the media used in this study will have been supplemented with tryptophan to allow growth. This however will not elicit the GAAC, as previous research has suggested that this amino acid alone cannot induce this response (Niederberger *et al*., 1981). In addition to this, Herzog *et al* only used three repeats when investigating the translation of the mRNA of *GCN4* in response to ER stress. Additionally, this study only independently reported on *GCN4-lacZ* activity and *GCN4Δ4uORF-lacZ* activity, without measuring translational efficiency. Therefore, the results of this current study suggesting that Gcn4 does not have an indirect role in the UPR may be more reliable than the conflicting evidence of these previous studies, as six repeats were used for each *S. cerevisiae* strain during the UPR and GAAC assays and the translational efficiency was also calculated. This current study additionally used a p180 plasmid containing the *GCN4-lacZ* reporter (Hinnebusch, 1985). The use of this reporter validates that the medium of SD - U used in this study was balanced as there were no significant differences in β-galactosidase/ protein (U/g) activity between all three *S. cerevisiae* strains (Fig 10 A). This again differs from the Patil study, as that study did not validate the composition of media used. It can be interpreted that the GAAC was however activated because the genotype of the WT strain used was *MAT a; ura3-1; leu2-3,-112; his3-11,-15; trp1-1; ade2-1; can1-100*, which required for example uracil, leucine, histidine, tryptophan and adenine supplementation for inactivated genes. Therefore, differences could be due to as previously mentioned leucine supplementation, without isoleucine and valine, causing GAAC activation, as these branched amino acids are involved in the biosynthesis of each other (KEGG, 2021; Wek *et al*., 1995).

7.3 Limitations and future directions of the work

The main result of this study was concluded from reporter assays utilising β-galactosidase activity, which was advantageous compared to the growth assay, as they gave a quantitative measurement of activity from each independent clone (Möckli and Auerbach, 2004). Protein interactions between Hac1 and Gcn4 were however not investigated, so could possibly be explored using a range of methods including co-immunoprecipitation (Co-IP), bimolecular fluorescence complementation (BiFC) and pull-down assays (Dong and Li, 2018; Iqbal *et al*., 2018; Shyu *et al*., 2006). On the other hand, this would only conclude that Hac1 and Gcn4 interact and not that they bind to UPREs as a heterodimer. Therefore, it would be suggested to perform confirmatory experiments of the data already obtained in this project by looking at both mRNA and protein synthesis rates. Methods involving quantitative RT-PCR (RT-qPCR) and liquid scintillation counting (LSC) of [^{35}S]-methionine labelled cells may be used to achieve this (Ho *et al*., 2018; Yaffe and Schatz, 1984). Investigating mRNA levels in terms of the splicing of *HAC1* mRNA and UPR target gene mRNA induction would be beneficial to directly measure outcomes from the UPR to determine if it is truly diminished. This would show that not only is there a reduction in reporter plasmid translation calculated from the ratio of β-galactosidase/ protein from the reporters of the Z691 and p227 plasmids, but that there is a reduction in the activation of the UPR. Further to this, the high-throughput method of mass spectrometry could also be utilised to provide a quantitative result of the provision of amino acids in the unbalanced medium, as the media composition was only implied in this study (Cooper *et al*., 2010). This also leads onto another limitation of this study that is associated with the provision of amino acids in the media. A generalisation of an amino acid

imbalance was made by creating just one specific type of imbalance of amino acids. Therefore, it would be of interest to investigate imbalances caused by other amino acids, not just leucine, and compare the results. Finally, as leucine, isoleucine and valine share various steps in their biosynthesis (KEGG, 2021), it would be useful to test that the balance in amino acids is restored from imbalance caused by supplementation with only leucine via supplementation with isoleucine and valine.

8.0 **Conclusion**

In conclusion, the results presented in this paper suggest that Gcn4 does not have a requirement during the UPR and that its role is to restore an amino acid balance during the GAAC. This study therefore has importance in suggesting that a heterodimer between Hac1 and Gcn4 does not form, which supports previous studies where only Hac1 exists as a transcription factor during the UPR (Fordyce *et al*., 2012; Mori *et al*., 1996).

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