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Identification of novel proteins that potentially are in complex with Yih1 and that are required for promoting Gcn2 function

A thesis submitted in partial fulfilment of the requirements
for the degree of Master of Philosophy in Biochemistry

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The ability of organisms to respond to various stress conditions is important for life. Under amino acid starvation conditions the protein Gcn2 is activated and phosphorylates the translation initiation factor eIF2α. This leads to a downregulation of general protein synthesis and an upregulation of the synthesis of proteins that are involved in helping the cell overcome starvation, a process called General Amino Acid Control (GAAC). It is important that the GAAC is only switched on when necessary and for this Gcn2 needs to be regulated. For instance, the protein Gcn1 is needed for Gcn2 activation. Another protein, Yih1, inhibits Gcn2 activity by competing with Gcn2 for Gcn1 binding. However, the balance between Gcn2 activation by Gcn1 and Gcn2 inhibition by Yih1 is not well understood. Actin was already identified as a Yih1-binding protein and modelling exercises strongly suggests that additional proteins bind Yih1.

The aim of this project was to identify novel proteins that are in a complex with Yih1 (Yih1-binding proteins, YBP) and to then discover which are required for Gcn2 activation. For the first aim, YBP were ascertained from published large-scale protein-protein interactions studies and from data generated in-house.

19 different strains deleted for one putative YBP exhibited an impaired growth under starvation conditions. Of those, four deletion mutants showed a reduced Gcn2 activity. One protein was Spc72 which is involved in mitochondrial organisation. Another protein was Idh2, an enzyme of the citric acid cycle. The growth defect of strains deleted for *SPC72* or *IDH2* was complemented with a plasmid containing *SPC72* or *IDH2*, respectively, and other genes. This suggested their involvement in Gcn2 activation.

Elongation factor eEF1A was found as a putative YBP and as a co-precipitator of Yih1, supporting previous unpublished observations. eEF1A was found to bind Gcn2 in previous studies and this suggested that Yih1-eEF1A interaction may regulate Gcn2 activation.

Another putative YBP, the heat shock protein Hsc82, is needed for Gcn2 maturation. Strains deleted for *HSC82* showed an impaired growth under starvation conditions and this was reversed by deleting *YIH1*. This suggested that Yih1 may regulate Hsc82-Gcn2 interaction and thus Gcn2 activity.

This study was a step to further advance our understanding of Yih1-binding proteins and Gcn2 activity. In addition, this further emphasised the idea of Yih1 as an important regulator inside the cell.

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List of abbreviations

3AT	3-amino-1,2,4-triazole
APS	ammonium persulfate
AGC	automatic gain control
ATP	adenosine triphosphate
CID	collision induced dissociation
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
eIF2	elongation initiation factor 2
F-actin	filamentous actin
FTMS	Fourier transform mass spectrometry
GAAC	general amino acid control
G-actin	globular (monomeric) actin
Gcn	general control non-derepressible
GDP	guanosine diphosphate
GST	glutathione S-transferase
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His_6	hexahistidine tag
HSP	heat shock protein
IMPACT	imprinted and ancient
IPTG	isopropyl β-D-1-thiogalactopyranoside
KCl	potassium chloride
kDa	kilodalton
LB	lysis broth
LC-MS/MS	liquid chromatography-tandem mass spectrometry
mRNA	messenger RNA
NaCl	sodium chloride
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PIC	pre-initiation complex
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RWD	RING finger, WD repeat, yeast DEAD-like helicase
SD	synthetic dextrose
SDS	sodium dodecyl sulfate

SM	sulfometuron methyl
SM^s	SM sensitivity
TAP	tandem affinity purification
TBS	Tris-buffered saline
TBS-T	TBS-Tween20
TOR	target of rapamycin
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
v/v	volume/volume
w/v	weight/volume
WCE	whole cell extract
YBP	Yih1-binding protein(s)
Yih1	Yeast Impact Homolog 1
YPD	yeast extract peptone dextrose
YPG	yeast extract peptone glycerol

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Chapter A

Introduction

Chapter A: Introduction

Every organism, be it single celled or multicellular, is exposed to a variety of stresses during its life. Examples of such stresses are viral infection, oxidative stress, high or low temperatures, UV irradiation, or nutrient limitations (Wek et al., 2006). Because of this they have evolved mechanisms to overcome a stress and survive. The response of an organism to a stress is designed to reduce damage to the cell or the organism. This response involves a complex network of proteins and is strictly regulated to ensure an appropriate response to all stresses. For example, protein synthesis depends on a steady supply of amino acids. One mechanism that helps the cell overcome nutritional stress in the form of amino acid deficiency is termed *General Amino Acid Control* (GAAC) (Delforge et al., 1975; Pierard et al., 1979; Wolfner et al., 1975).

A.1 Protein translation is a cyclical process consisting of initiation, elongation and termination

The synthesis of proteins takes place on ribosomes in three phases: 1. Initiation of proteins synthesis, 2. elongation of the peptide chain by incrementally adding amino acids, and 3. termination which ends the protein synthesis and releases the peptide chain from the ribosome (Figure A1).

At the initiation step a protein called eukaryotic initiation factor 2 (eIF2), GTP (guanosine-5'-triphosphate) and the initiator methionyl-tRNA ($tRNA_i^{Met}$) come together to form the ternary complex (Hinnebusch and Lorsch, 2012; Jackson et al., 2010). This complex is docking to the small (40S) ribosomal subunit and together with eIF1, 1A, 3 and 5 it forms the 43S pre-initiation complex (PIC). The PIC binds the mRNA near the 5′-7-methyl-guanosine (5′-m7G) cap, forming the 48S complex, and proceeds to scan in a 5’ to 3’ direction. Upon recognising the start codon (AUG) the initiation factors that are part of the PIC are released (eIF1, 1A, 2, 3 and 5). Simultaneously, eIF5B promotes the joining of the 60S ribosomal subunit and the PIC to form the 80S initiation complex. This allows protein elongation to begin.

eIF2 is released in an inactive, GDP-bound form upon recognising the start codon. Another protein, the guanosine exchange factor eIF2B, recycles GDP to GTP and allows eIF2 to act in another initiation cycle.

At the elongation step eukaryotic elongation factor 1A (eEF1A), GTP and aminoacyl-tRNA (charged tRNA) come together to form a ternary complex, thereby guiding the aminoacyl-tRNA to the A site of the 80S initiation complex (Dever and Green, 2012). Following this, eEF1A dissociates and GDP is recycled to GTP by its guanosine exchange factor eEF1B. The A site-bound aminoacyl-tRNA forms peptide bonds with the P site-bound peptidyl-tRNA. Another elongation factor, eEF2, is facilitating the translocation of tRNAs from the A to the P site and from the P to the E site, respectively (Spahn et al., 2004). While the exact details remain to be determined (Chen et al., 2011; Nierhaus and Pech, 2012; Petropoulos and Green, 2012) a reciprocal relationship appears to exist between the A and E sites: As the deacyl-tRNA is released from the E site the A site is again available to bind aminoacyl-tRNA, allowing for another elongation cycle. In addition to eEF1 and eEF2 another elongation factor, eEF3, is only found in several fungal species (including yeast) and is not present in other pro- or eukaryotes (Dever and Green, 2012). Its proposed function is in releasing deacyl-tRNA from the E site (Andersen et al., 2006; Triana-Alonso et al., 1995). Interestingly, eEF3 is known to interact with eEF1A, supporting a model in which eEF3 assists eEF1A in the delivery of aminoacyl-tRNA to the A site (Anand et al., 2003, 2006).

The elongation cycle repeats until the release factor eRF1 recognises the stop codon (Dever and Green, 2012). A second release factor, eRF3, binds eRF1 and stimulates its activity in a GTP-dependent manner. The eRF1-eRF3-GTP complex binds the ribosome at the A site, GTP is hydrolysed and eRF3 dissociates. The ATPase ABCE1 then binds eRF1 and this induces the polypeptide chain-release by eRF1 (Preis et al., 2014; Shoemaker and Green, 2011). Interestingly, the dissociation of mRNA, tRNA and the ribosomal subunits (ribosome recycling) is catalysed by eEF3 (and ATP) (Kurata et al., 2010, 2013). For some genes the scanning 40S ribosomal subunit remains attached to the mRNA which allows reinitiation of protein translation (see next section).

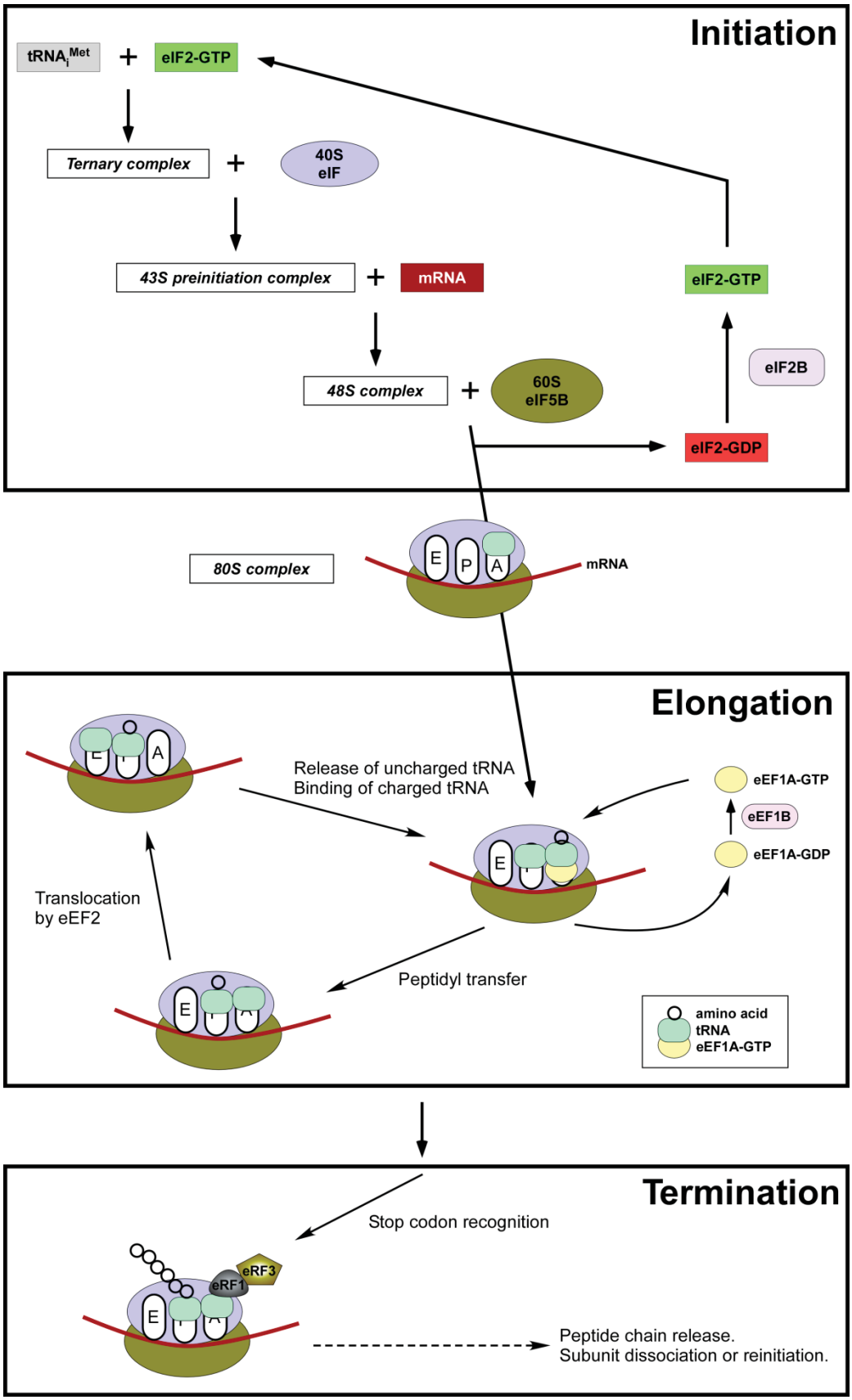


Figure A1: Schematic overview of protein translation. The positions of tRNAs, mRNA and factors are only indicative and not meant to show their exact locations on the ribosome. For details see text. Adapted from Dever & Green 2012 and Hinnebusch & Lorsch 2012.

A.2 Translational control by phosphorylation of initiation factor eIF2 and the regulatory role of the uORFs in *GCN4* mRNA

The GDP-to-GTP recycling of eIF2 can be inhibited by phosphorylation of a conserved serine residue in its α subunit (eIF2α-P) (Dever et al. 1995). This serine residue is Ser51 in *Saccharomyces cerevisiae*, or budding yeast, the organism used in this study. eIF2α-P is a potent inhibitor of eIF2B as it remains tightly bound to eIF2α-P and this abrogates eIF2B activity. The result is a reduced level of ternary complex and subsequently a reduced level of protein synthesis. At the same time, a selective increased translation of *GCN4* mRNA occurs. Gcn4 is a transcriptional activator and targets genes coding for proteins involved in amino acid biosynthesis, as well as vitamin biosynthetic enzymes, autophagy proteins and more (Jia et al. 2000; Natarajan et al. 2001). Consequently, this allows cells to survive amino acid starvation stress.

GCN4 mRNA contains four upstream ORFs (uORF1-4) that are important for regulating *GCN4* expression (Hinnebusch, 2005). Under replete conditions, when sufficient amounts of amino acids are present, the PIC binds the *GCN4* mRNA 5' cap. After recognising the first starting codon, the AUG of uORF1, the PIC and the 60S subunit form the 80S complex and translate uORF1. After termination upon reaching the stop codon of uORF1 the 60S subunit and the ternary complex dissociate but most of the scanning 40S subunits remain attached to the mRNA, allowing them to rebind a ternary complex. This results in a reinitiation at one of the downstream uORFs (Figure A2). However, after translation of these uORFs both ribosomal subunits dissociate fully and *GCN4* is not translated and remains repressed. Under starvation conditions the level of ternary complex is low due to a reduced number of aminoacyl-tRNA. Under these conditions the scanning 40S are less likely to reinitiate after translating uORF1 because the distance between uORF1 and uORF4 is not long enough to reacquire a ternary complex. The 40S subunits are more likely to bypass the inhibitory uORFs 2 to 4 and to reinitiate at the *GCN4* start codon.

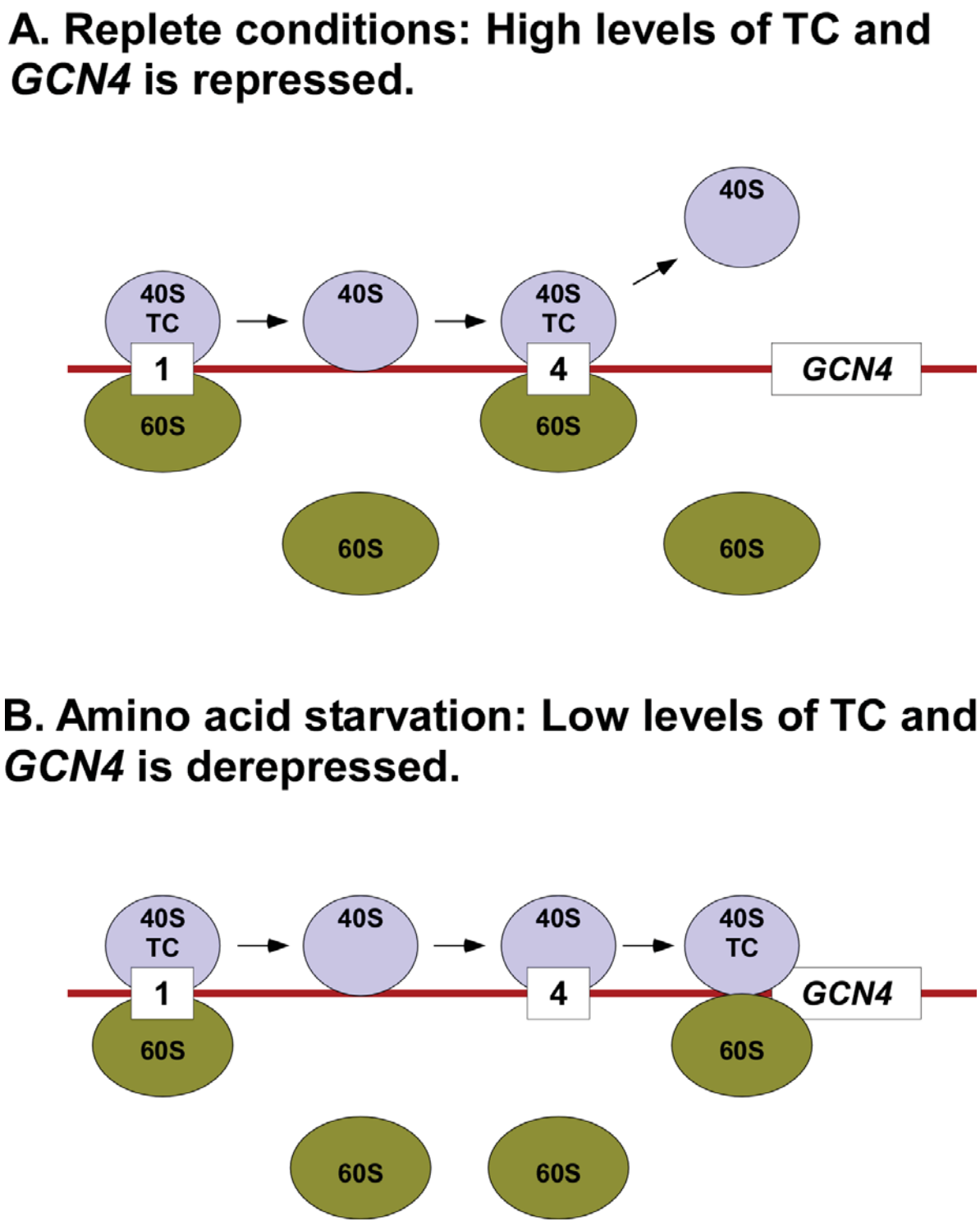


Figure A2: Schematic representation of *GCN4* expression control under replete (A) and amino acid starvation (B) conditions. *GCN4* mRNA is depicted with uORFs 1 and 4 and the *GCN4* coding sequences shown as boxes. uORF2 and 3 are functionally redundant with uORF4 and were omitted. TC: Ternary complex. Arrows indicate the movement of the scanning complex along the mRNA. Not to scale. For details see text. Adapted from Hinnebusch 2005 and Munzarová et al. 2011.

A.3 The eIF2α kinase Gcn2 is essential for survival under amino acid starvation

In mammals, the family of protein kinases that phosphorylate eIF2α consists of four proteins (Wek et al., 2006): HRI (heme regulator inhibitor), PKR (RNA-dependent protein kinase), PERK (PKR-like endoplasmic reticulum kinase), and Gcn2 (general control non-derepressible). Each protein is sensitive to a different type of stress and this helps the cells to respond to a variety of adverse events (Wek et al., 2006): HRI is activated under heme deficiency and oxidative or heat stress. PKR is activated during viral infections. PERK is activated by unfolded proteins in the endoplasmic reticulum. Gcn2 is activated under amino acid starvation, UV irradiation or acid stress (Hueso et al., 2012).

Gcn2 is the only eIF2α kinase that exists in *Saccharomyces cerevisiae* (Dever, 2002). Gcn2 consists of five domains (from N- to C-terminus, Figure A3): The N-terminus consists of the RWD domain (previously called GI domain (Kubota et al., 2000)), so named after its presence in RING finger, WD repeat and DEAD-box like proteins (Doerks et al., 2002). The RWD domain binds to another protein, Gcn1 (see below in the next section). Next are a pseudokinase which may interact with and activate the kinase domain upon tRNA binding to HisRS (Lageix et al., 2014), a protein kinase domain which phosphorylates eIF2α, a HisRS-like (histidyl-tRNA synthetase) domain able to bind uncharged tRNA and a C-terminal domain which has ribosome-binding and dimerisation functions (Hinnebusch, 2005). Gcn2 must function as a dimer but is shown in figures in this work only as a monomer (Qiu et al., 1998).

Gcn2 is essential for cell survival under amino acid starvation conditions and this was shown by several lines of evidence. Deletion of the *GCN2* gene in yeast does not lead to a growth defect under nutrient rich conditions; however, under amino acid starvation these yeast strains are unable to activate the GAAC and therefore show a strong growth defect (Hinnebusch, 1988). This is called a Gcn^- phenotype. Studies have shown that Gcn2 is a positive regulator of Gcn4 under starvation conditions: In yeast strains deleted for *GCN2* the expression of the transcriptional activator *GCN4* remains low (Hinnebusch, 1994). Additionally,

overexpression of *GCN4* can partially revert the growth defect of strains without Gcn2 (Hinnebusch and Fink, 1983).

It was shown that Gcn2 is inactive due to intramolecular autoinhibitory interactions which are relieved by binding with other proteins or tRNA (Hinnebusch, 2005). In fact, certain single mutations in the kinase domain, the HisRS-like domain or the C-terminus of Gcn2 can render it constantly active (constitutively active) and subsequently reduce protein synthesis due to increased levels of eIF2α-P (Ramirez et al., 1992).

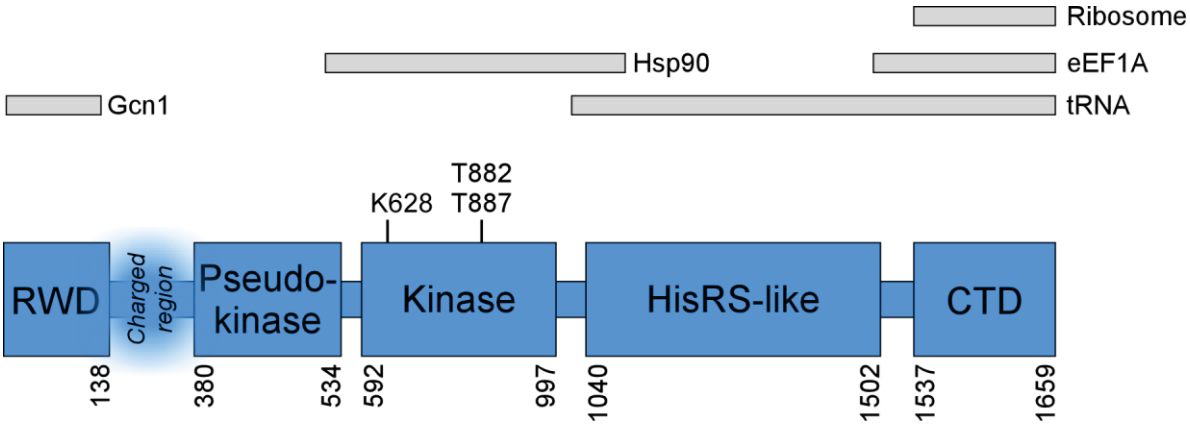


Figure A3: Schematic overview of the monomeric Gcn2 protein. The binding areas of important binding partners are indicated with gray boxes. Several amino acids with known functions are indicated. K628 is crucial for the kinase catalytic activity as mutating this residue abolishes auto-phosphorylation and eIF2α phosphorylation (Wek et al. 1989). T882 and T887 are auto-phosphorylation sites that are required for Gcn2 activation (Romano et al., 1998). Not to scale. Adapted from Castilho et al. 2014 and amino acid positions are taken from Lageix et al. 2015.

A.4 Significance of this study

Why should we be interested in Gcn2? In yeasts Gcn2 is known to be involved in helping cells overcome diverse stresses (Cambiaghi et al., 2014; Donze and Picard, 1999; Goossens et al., 2001; Parsons et al., 2004). This still holds true in mammals (Wek et al., 2006) but Gcn2 has acquired additional functions apart from the stress response as required by multicellular organisms: It can sense amino acid-scarcity in the diet and lead to behavioural changes to avoid such deficient food (Anthony et al., 2004; Hao et al., 2005; Koehnle et al., 2003; Maurin et al., 2014). At the same time it is required for adaptation to amino acid deprivation and preservation of protein synthesis in the liver (Anthony et al., 2004;

Harding et al., 2000; Zhang et al., 2002). It plays a role in neuronal cell development and is crucial for long term memory (Costa-Mattioli et al., 2005; Roffé et al., 2013; Trinh and Klann, 2013). Gcn2 function was implicated in a number of diseases and disorders, for example it may play a role in cancer cell survival (Wang et al., 2013; Ye et al., 2010), obesity (Maurin et al., 2005), auto-immune disease (Cosnefroy et al., 2013) or even neurodegenerative disorders such as Alzheimer's (Devi and Ohno, 2013; Ma et al., 2013). Research suggests that Gcn2 is involved in the response of the immune system against viral infections or tumour cell growth (Berlanga et al., 2006; Brenk et al., 2009; Fallarino et al., 2006; Forouzandeh et al., 2008; Hirohata et al., 2015; Muller et al., 2008; Tong et al., 2011; Won et al., 2012).

How can Gcn2 be involved in so many different functions? How can the cell ensure that Gcn2 is always performing the right function for a particular input or a specific cell type? It is clear that a complex regulatory pathway must be involved. However, our knowledge of Gcn2 regulation remains limited. It is known that Gcn2 needs to bind to another protein, Gcn1, in order to be activated (see section A.5). However, one important aspect that needs to be better understood is how Gcn2 is negatively regulated. So far, the yeast protein Yih1 is known as a Gcn2 inhibitor (see section A.9). Thus, advancing our understanding of Yih1 regulation can then advance our understanding of how Gcn2 activity is promoted. Gcn2, Gcn1 and Yih1 proteins are highly conserved among eukaryotes and therefore the model organism *Saccharomyces cerevisiae* is used as a starting point to understand this regulatory pathway in mammals.

A.5 Gcn2 activity is dependent on its interaction with Gcn1

The process of eIF2 phosphorylation by Gcn2 involves two additional proteins: Gcn1 (Figure A4) and Gcn20. Similar to Gcn2, Gcn1 and Gcn20 are not essential for cell survival under replete conditions (Marton et al., 1993; Vazquez de Aldana et al., 1995). Under amino acid starvation conditions, however, Gcn1 is needed for Gcn2 activation. Under these conditions yeast cells deleted for *GCN1* showed reduced growth and no eIF2α phosphorylation (Marton et al., 1993). At the same time, deletion of *GCN1* does not affect the intrinsic kinase activity of Gcn2 and this indicated that Gcn1 might be specifically involved in sensing the

starvation signal (Marton et al., 1993). In comparison to Gcn1, deletion of the *GCN20* gene only leads to a reduced cell growth rate under starvation conditions and a reduction in the level of eIF2α-P (Vazquez de Aldana et al., 1995). This suggested that Gcn20 is not essential for Gcn2 activity and that it may act as an attenuator of Gcn1 and thus Gcn2 function.

Using Gcn2 fragments (expressed from high copy plasmids) in co-immunoprecipitation assays allowed more in-depth studies on its interaction with Gcn1. Removing the N-terminus of Gcn2 (amino acids 1-125) resulted in reduced binding of Gcn1 *in vivo* while the deletion of other regions did not influence Gcn1 binding (Garcia-Barrio et al., 2000). In addition, a mutation in Gcn2 in the kinase domain that reduced its kinase activity did not reduce Gcn1-Gcn2 interaction. This showed that Gcn2's RWD domain is the determinant for Gcn1 binding. These authors showed that Gcn2 fragments 10-109 and 110-235 have the ability to interact with Gcn1. Another study used yeast two-hybrid studies to show that a Gcn2 fragment consisting of amino acids 1-125 was sufficient and essential for Gcn1 binding although fragment 126-272 showed no interaction (Kubota et al., 2001). These findings were consistent with the idea that Gcn2 uses its RWD domain to bind Gcn1. The RWD consists of approximately the first 129-134 amino acids in Gcn2 (Nameki et al., 2004; Sattlegger et al., 2011). Thus, the 110-235 fragment may contain Gcn1-binding determinants while the 126-272 did not. Furthermore, in the study by Garcia-Barrio et al. (2000) it was shown that upon deletion of the N-terminal 538 amino acids the amount of eIF2α-P is reduced, supporting the idea that the RWD domain of Gcn2 binds Gcn1 and that this interaction is needed for proper eIF2α phosphorylation.

A.6 Gcn2-Gcn1 interaction depends on a single amino acid in Gcn1

To identify which Gcn1 region binds to Gcn2, Gcn1 fragments were used in a similar fashion as was done when Gcn2 fragments were used (Sattlegger and Hinnebusch, 2000). Gcn1 is a large protein consisting of 2672 amino acids. A fragment encompassing residues 2052 to 2428 of Gcn1 showed the strongest Gcn2 interaction of all fragments tested, suggesting that the Gcn2-binding determinant is located in the C-terminus of Gcn1. Removing amino acids 2052 to 2428 from Gcn1 impaired growth under amino acid starvation conditions induced

by 3AT (3-amino-1,2,4-triazole, inhibits histidine biosynthesis) and this further indicated that this region is needed for Gcn2 activation (Sattlegger and Hinnebusch, 2000).

Strikingly, the interaction between Gcn2 and Gcn1 is dependent on a single amino acid in Gcn1: A point mutation was introduced into the Gcn1 fragment that contains the Gcn2-binding determinant. This substitution of arginine with alanine at position 2259 (R2259A) significantly reduced the level of Gcn2-Gcn1 interaction, both *in vitro* and *in vivo* (Sattlegger and Hinnebusch, 2000). Furthermore, overexpression of this Gcn1 mutant in the wild type strain caused a strong cell growth defect under amino acid starvation conditions. This indicated that its overexpression prevented the interaction of wild type Gcn1 and Gcn2 and thus prevented activation of the GAAC. Consequently, overexpression of Gcn2 can revert the growth defect and restore wild type growth, likely because increased levels of Gcn2 can compensate for the reduced Gcn2-binding affinity of the Gcn1 mutant. Taken together this shows the importance of this single amino acid residue in activating Gcn2 and enabling cells to overcome amino acid starvation.

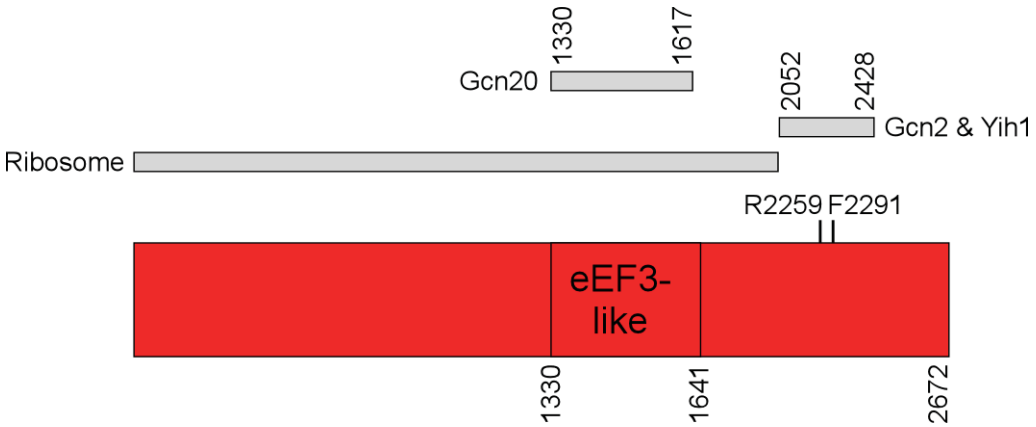


Figure A4: Schematic overview of Gcn1. Indicated are the binding regions of ribosomes, Gcn2, Yih1 and Gcn20. R2259 is required for Gcn2 and Yih1 binding *in vivo* and *in vitro* while F2291 is required for Gcn2 binding *in vivo*. Not to scale.

A.7 Uncharged tRNA is the signal that activates Gcn2

How can Gcn2 recognise the starvation stress? A first clue came from the observation that the RelA protein in *E. coli* can sense uncharged tRNA and induce the amino acid starvation response (Goldman and Jakubowski, 1990). As Gcn2 contains a domain with tRNA-binding features, the HisRS domain, this suggested

a similar mechanism as for RelA (Marton et al., 1997). Another clue came from the observation that both Gcn1 and Gcn20 contain regions with similarity to EF3 (Marton et al., 1993). eEF3 is an elongation factor that is proposed to stimulate the release of deacylated (uncharged) tRNAs from the ribosomal E site. This facilitates the delivery of additional aminoacyl-tRNA to the A-site due to a reciprocal linkage between the two sites (Triana-Alonso et al., 1995). As the starvation signal inside a cell is uncharged tRNA it was proposed that Gcn1 and Gcn20 are transmitting the starvation signal to Gcn2 (Marton et al., 1997; Sattlegger and Hinnebusch, 2000). A Gcn1/2/20 trimeric complex may promote binding of uncharged tRNA to the A site of translating ribosomes (polysomes). Another possibility is that the Gcn1/Gcn20 complex transfers uncharged tRNA to the tRNA-binding domain HisRS of Gcn2 (Figure A5). Alternatively, Gcn1 may act as a scaffolding protein to hold Gcn2 in a conformation that allows other proteins to transfer tRNA to HisRS, allowing for eIF2α phosphorylation (Hinnebusch, 2005; Sattlegger and Hinnebusch, 2000). However, the exact mechanism of how the tRNA is transferred to Gcn2 is not known at this time.

A.8 Gcn1 needs ribosome binding to positively regulate Gcn2

Just like Gcn2, Gcn1 binds to the ribosome and this interaction is required for Gcn2 activity (Marton et al., 1997). Ribosomes exists in 40S (small subunit), 60S (large subunit), 80S (full ribosome) forms as well as in clusters of translating ribosomes (polysomes). One approach to verify if there is a Gcn1-ribosome association is to use velocity sedimentation of whole cell extracts in a sucrose gradient. The individual forms of ribosomes are separated by their velocity, fractions are collected and the proteins that are present in each fraction are identified using western blotting. Using this approach it was found that Gcn1 and Gcn2 co-sediment with polysomes (Marton et al., 1997; Ramirez et al., 1991). In addition, the use of Gcn1 fragments showed that deleting either the central part of Gcn1 (which includes the EF3-like domain) or the N-terminus reduced polysome association (Sattlegger and Hinnebusch, 2000). This indicated that the N-terminal three quarters of Gcn1 is needed for ribosome binding.

In order to study if Gcn1 binding to the ribosome affects Gcn2 activity mutations were introduced into the ribosome-binding regions of Gcn1 (Sattlegger

and Hinnebusch, 2005). These reduced the ribosome association of Gcn1 and subsequently impaired the starvation response, as measured by growth assays under amino acid starvation conditions. There is evidence that this is due to a reduced level of Gcn2 activity as shown by a reduced level of eIF2α phosphorylation (Sattlegger and Hinnebusch, 2005). This indicated that full Gcn2 activation is dependent on Gcn1-ribosome interaction.

In bacteria, the drug paromomycin binds the A site of the small ribosomal subunit and affects translational fidelity and proofreading (Eustice and Wilhelm, 1984; Palmer et al., 1979; Vicens and Westhof, 2001). These effects appear to be conserved among pro- and eukaryotes (Fan-Minogue and Bedwell, 2008). When there is no Gcn1 present in yeast then these cells were more resistant to paromomycin than the wild type (Sattlegger and Hinnebusch, 2000). In addition, overexpression of Gcn1 conferred an increased sensitivity to this drug compared to the wild type.

It is possible that Gcn1 binding to the ribosome can induce a conformational change to the A-site that makes cells susceptible to paromomycin. These results suggested that Gcn1 binds at or near the A-site and this may allow it to sense and transmit uncharged tRNA to the A-site or to Gcn2 (Figure A5); however, this has not been verified yet (Hinnebusch, 2005; Sattlegger and Hinnebusch, 2000).

A.9 Yih1, the negative regulator of Gcn2

As we have seen Gcn1 is the positive regulator of Gcn2. In contrast, the protein Yih1 in budding yeast and its mammalian homolog IMPACT are negative regulators of Gcn2 activity.

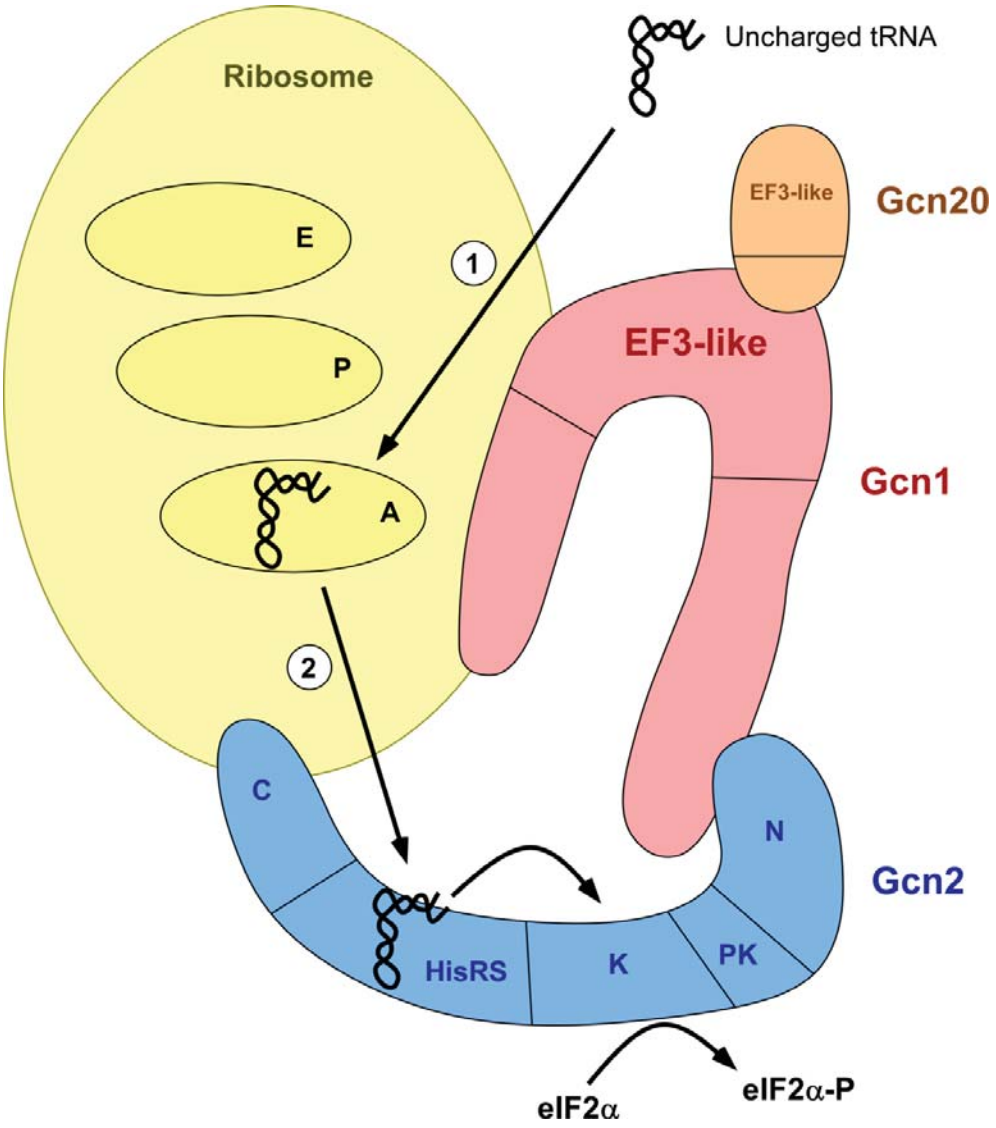


Figure A5: Proposed model of the role of the Gcn1/Gcn20 complex in activating Gcn2 via uncharged tRNA at the ribosomal A site. Gcn1/20 may facilitate the binding of uncharged tRNA to the A site (arrow 1) or transfer uncharged tRNA from the A site to the HisRS domain in Gcn2 (arrow 2). For more details see text. Gcn2 domains are as follows: C, N: C- and N-terminus, respectively. K: Kinase domain. PK: Pseudokinase domain. Not to scale. Adapted from Hinnebusch 2005.

Impact was first discovered as an imprinted gene[1] in mice and was later found to be conserved among eukaryotes (Hagiwara-Takeuchi et al., 1997; Okamura et al., 2000; Yamada et al., 1999). Proteins with partial homology to the product of *Impact* exist in prokaryotes but the homology is restricted to the central and C-terminal region of the protein. The baker's yeast homolog of the *Impact* gene product is called Yih1 (yeast IMPACT homolog), a protein with a length of

[1] An imprinted gene is a gene whose expression is determined in a parent-of-origin manner. Thus, one allele is active while the other allele is silenced in the daughter cell.

258 amino acids (Kubota et al., 2000; Sattlegger et al., 2004). This protein will be the focus of this study. Yih1 consists of three domains: An N-terminal RWD domain and a C-terminal "ancient domain", connected by a flexible linker region (Figure A3) (Sattlegger et al., 2011). The RWD domain is only found in eukaryotes and it is missing in prokaryotes. Since the RWD domain is also present in Gcn2 this suggested a possible interaction of Yih1 with Gcn1 (see below in section A.10). The ancient domain contains conserved amino acid residues and is ubiquitous in all life forms (Okamura et al., 2000; Sattlegger et al., 2011). For instance, it has homology to the N-terminal half of the YigZ family of prokaryotic proteins of unknown function (Park et al., 2004; Sattlegger et al., 2011). However, the function of the ancient domain remains unclear. As the ancient domain contains conserved residues this suggests that this domain may bind to an evolutionarily conserved, but as yet unknown, protein or molecule (Green et al., 1993; Sattlegger et al., 2011).

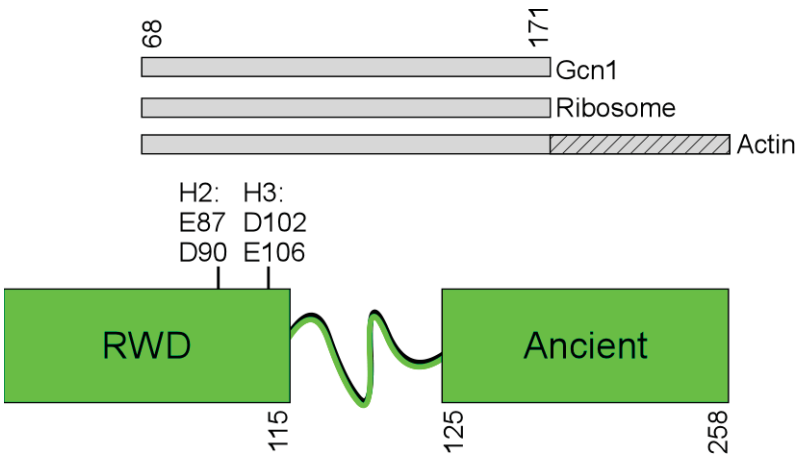


Figure A6: Schematic overview of Yih1 (yeast IMPACT) and its domains. The approximate location of the double point mutations in helix 2 and 3 in the RWD domain are indicated and are discussed in section A.13. Not to scale.

A.10 Yih1 overexpression reduces Gcn2 activity

Initial experiments showed that Yih1 at overexpressed levels inhibits the growth of cells under starvation conditions (Kubota et al., 2000). Later studies investigated the effects of overexpressed Yih1 on the GAAC in more detail: Under amino acid starvation conditions (induced by 3AT) cells overexpressing Yih1 were unable to grow (Sattlegger et al., 2004). Additionally, the overexpression of Yih1 significantly reduced the level of eIF2α phosphorylation under both starved and

unstarved conditions. This gcn$^-$ phenotype was suppressed by overexpressing Gcn2. As discussed above a constitutively active Gcn2 (Gcn2^C) resulted in slow growth due to a reduced protein synthesis. This growth defect was partly overcome by simultaneously overexpressing Yih1, showing that Yih1 can inhibit the Gcn2-mediated starvation response (Sattlegger et al., 2004).

As excess amounts of Yih1 reduce the activity of Gcn2 it was conceivable that the absence of Yih1 would result in increased levels of Gcn2 activation. A first step to test this was to expose wild type (*YIH1*$^+$) cells to amino acid analogues 5-fluoro-DL-tryptophan (5FT) or triazolealanine (TRA). These molecules render cells slow growing due to an impaired protein synthesis and this should be overcome by deleting *YIH1* (Sattlegger et al., 2004). However, this was not the case and there was no growth difference compared to the wild type cells (Sattlegger et al., 2004). Furthermore, strains containing the constitutively active Gcn2^C which renders the GAAC constantly active are resistant to amino acid analogues (Ramirez et al., 1992). Thus, if Yih1 is inhibiting Gcn2 then deletion of Yih1 may result in an active Gcn2 and wild type growth when cells are exposed to amino acid analogues. However, *YIH1* deletion strains containing the constitutively active Gcn2^C showed no growth difference compared to cells without Gcn2^C (Sattlegger et al., 2004). Additionally, eIF2α-P levels were not affected in *YIH1* deletion strains under both non-starvation and starvation conditions (Sattlegger et al., 2004).

To explain why Yih1 was only found as a Gcn2 inhibitor when it was overexpressed it was speculated that Yih1 inhibits Gcn2 only under certain conditions, at a certain cell cycle stage or in localised areas of the cell (Sattlegger et al., 2011). One possible example would be the growing bud tip in yeast where a high level of protein expression is required: Hypothetically, Yih1 could prevent Gcn2 activation in this region and ensure that protein synthesis remains sufficiently high for continued cell growth (Sattlegger et al., 2011). Only one study investigated the subcellular localisation of Yih1 and found it in the nucleus and cytoplasm but without differentiating further between cytosol and organelles and more studies are required to verify these findings (Huh et al., 2003). No further studies have been published so far specifically on the subcellular location of Yih1 that could give insights into its function.

A.11 Yih1 competes with Gcn2 for Gcn1 binding

How does Yih1 inhibit Gcn2 function? As was mentioned above the presence of an RWD domain in Yih1 suggested that it binds to Gcn1, preventing Gcn1 from binding to and activating Gcn2 (Kubota et al., 2000). This idea is supported by initial yeast two-hybrid experiments which showed Gcn1 as a potential Yih1-binding protein (Kubota et al., 2000). Further support for this hypothesis came from *in vitro* studies which allowed the identification of Gcn1 fragments that interact with Yih1. These experiments showed that a Gcn1 fragment consisting of amino acids 2052-2428 was sufficient to co-precipitate Yih1 (Sattlegger et al., 2011).

In vivo studies provided additional evidence for a Yih1-Gcn1 interaction. Overexpressed Yih1 specifically co-precipitated a natively expressed Gcn1 (Sattlegger et al., 2004). The Gcn1[2052-2428] fragment with a mutation of Arg2259 into alanine reduces the interaction with Yih1 in these experiments, as seen for Gcn2-Gcn1 interaction.

To test how the Gcn1-Yih1 interaction affects the interaction of Gcn1 with Gcn2, Gcn1 was immunoprecipitated from whole cell extracts (Sattlegger et al., 2004). Gcn2 co-precipitated with Gcn1 when Yih1 was at native levels but overexpression of Yih1 reduced the amount of Gcn2 that was pulled down by Gcn1 by almost 50%.

Taken together these findings showed that Yih1 and Gcn1 interact and that this interaction depends on Arg2259. Yih1 overexpression reduced Gcn1-Gcn2 interaction and this suggested that Gcn2 and Yih1 share the same binding determinant in Gcn1 and that Yih1 can inhibit Gcn2 by binding to Gcn1 (Figure A7) (Sattlegger and Hinnebusch, 2005).

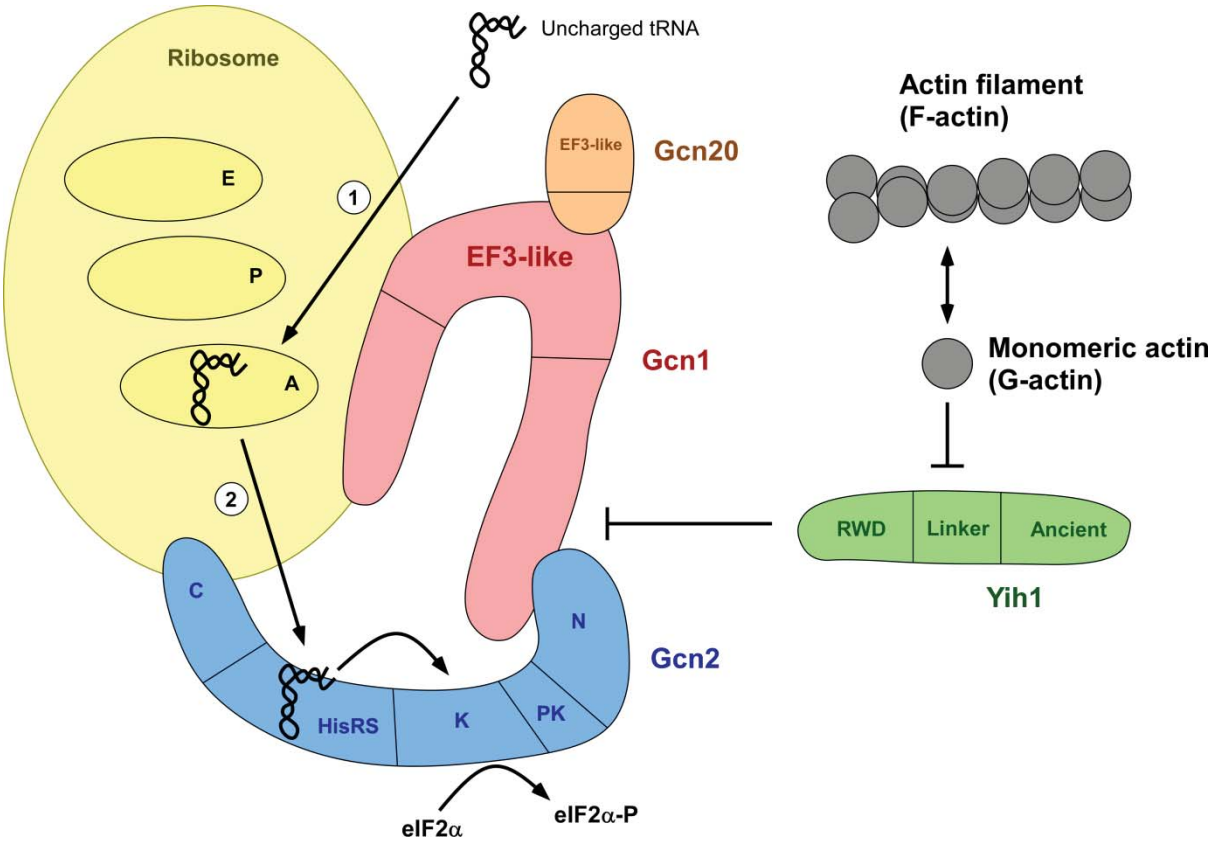


Figure A7: Model of the inhibitory effect of Yih1 on Gcn2 activity. The RWD domain of Yih1 binds Gcn1 and this inhibits Gcn2 activation. For details see text and Figure A5 legend. Not to scale. Adapted from Waller et al., 2012 and Castilho et al., 2014.

A.12 The RWD domain of Yih1 binds Gcn1

In order to verify if the RWD domain of Yih1 is indeed the domain that interacts with Gcn2, a set of different Yih1 fragments at overexpressed levels was used (Sattlegger et al., 2011). These fragments were investigated for their ability to co-precipitate Gcn1. Fragments consisting of the RWD domain and/or the linker region (spanning amino acids 1-171) showed significantly stronger Gcn1 binding than full length Yih1. The N-terminal region of the RWD domain (the first 67 amino acids) is dispensable or may actually restrict binding to Gcn1 (Sattlegger et al., 2011). This region contains a conserved YPXXXP motif which is also present in Gcn2. Interestingly, Kubota et al. reported that mutations in this motif abolished Gcn1-Yih1 and Gcn1-Gcn2 interactions, indicating that this region may be involved in Gcn1 binding (Kubota et al., 2000). To explain this discrepancy, it was suggested that the mutations cause structural instability in Yih1 and abolish its stable expression (Nameki et al., 2004; Sattlegger et al., 2011). The C-terminal

fragment of Yih1, which contains the ancient domain, showed almost no Gcn1 binding, indicating that it is not important for Gcn1-Yih1 interaction (Sattlegger et al., 2011). In fact, it may actually hinder the interaction with Gcn1 because the N-terminal fragment binds more Gcn1 than the wild type Yih1 (Sattlegger et al., 2011). Thus, the region in Yih1 that appears to be sufficient for Gcn1 binding consists of the RWD domain and a partial linker (corresponding to amino acids 68-132) (cf. Figure A6).

If the RWD domain of Yih1 binds Gcn1 then overexpressing an N-terminal Yih1 fragment containing the RWD domain should prevent Gcn2 activity. Thus, yeast cells would be unable to grow under starvation conditions and would be unable to phosphorylate eIF2α. This was the case: Only the Yih1 fragments consisting of amino acids 2-171 and 2-132 resulted in an impaired starvation response that was similar to overexpressed wild type Yih1 and a significant decrease in eIF2α-P levels (Sattlegger et al., 2011). These results are consistent with another study by Kubota et al. (2000). These authors reported that the overexpression of an N-terminal fragment of Yih1 (amino acids 1-125) impaired the starvation response. This further supported the idea that Yih1's RWD domain is involved in Gcn1 binding. Furthermore, these results further indicated that only part of the RWD alone is needed for Gcn1 binding and that the flexible linker has an important role to play as well.

A.13 Helix 3 in the RWD domain of Yih1 contains important Gcn1-binding determinants

The RWD domain of Gcn2 consists of three α-helices and four β-sheets in the order of αββββαα (Nameki et al., 2004). Glutamate residues in helix 3 were proposed to be needed for its Gcn1 interaction based on their conservation and exposure to the solvent (Nameki et al., 2004; Sattlegger and Hinnebusch, 2000). One study tested if these conserved residues are also present in Yih1 and if they are needed for Gcn1-interaction (Sattlegger et al., 2011). As a first step the sequences of the RWD domain from Yih1 and Gcn2 were aligned (Sattlegger et al., 2011). Subsequently, the structure of the Yih1 RWD domain was modelled using the published Gcn2 RWD structure as a template (Nameki et al., 2004;

Sattlegger et al., 2011). This revealed the presence of solvent-exposed glutamate residues in helices 2 and 3 of Yih1 (cf. Figure A6). To test if they act as docking sites for Gcn1 point mutations were introduced into these sites (Sattlegger et al., 2011). If Gcn1-Yih1 interaction depends on these amino acids then a mutation should decrease this interaction and reduce Yih1-mediated Gcn2 inhibition. In other words, Gcn1 is free to bind Gcn2 in strains expressing the mutated Yih1 and these strains should be able to overcome amino acid starvation and grow as the wild type. For one set of mutations Asp102 and Glu106 in helix 3 of Yih1 (Yih1*H3) were replaced with alanine (Sattlegger et al., 2011). A strain overexpressing Yih1*H3 can overcome amino acid starvation while strains overexpressing wild type Yih1 showed a growth defect. In addition, strains containing Yih1*H3 exhibited a decreased Yih1-Gcn1 interaction. This indicated that helix 3 in Yih1 contains determinants for Gcn1 binding (Sattlegger et al., 2011). In another set of mutations, Glu87 and Asp90 in helix 2 of Yih1 (Yih1*H2) were replaced by alanine. This strain presented a stronger growth defect than a strain with wild type Yih1 and Yih1*H2 co-precipitated a larger amount of Gcn1. This could indicate a conformational change in Yih1 caused by the mutation that allows Gcn1 better access to its binding site in helix 3 (Sattlegger et al., 2011). A strain overexpressing a Yih1 with mutations in helix 2 and 3 (Yih1*H2*H3) did not show starvation sensitivity and Yih1*H2*H3 co-precipitated less Gcn1 compared to the wild type Yih1, again indicating that helix 3 is needed for Gcn1 binding. Taken together, this suggests that the Gcn1 binding determinant lies within helix 3 in the C-terminus of the Yih1 RWD domain and that the interaction is determined by Asp102 and/or Glu106. This is supported by the observation that helix 3 lies within the minimal Yih1 fragment needed for Gcn1 binding (amino acids 68-132, see section 1.10). As the mutations in helix 3 did not fully abolish Gcn1 binding, additional binding determinants in Yih1 might exist.

The interaction between Yih1 and Gcn1 has only been observed when Yih1 was overexpressed, either *in vivo* or *in vitro*, but not when it was at native levels (Sattlegger et al., 2004, 2011). One explanation for this is that their interaction might be below the detection limit due to the relatively low expression level of Yih1 (~3,030 molecules per cell (Ghaemmaghami et al., 2003)) and Gcn1 (~7,330 molecules). Alternatively, the *in vivo* interaction presumably was too weak to withstand the purification process but no *in vitro* experiments with Yih1 at native

level were done (Sattlegger et al., 2004). Furthermore, the growth conditions (rich media and native level Yih1) may not have been conducive to Gcn1-Yih1 interaction (Sattlegger et al., 2004). Finally, it is possible that Yih1-Gcn1 interaction only occurs under specific conditions, for example when Gcn2 activity needs to be reduced (Sattlegger et al., 2004, 2011).

A.14 Actin is a Yih1-binding protein

Actin is another protein that was found to bind Yih1 (Sattlegger et al., 2004). The Yih1-actin interaction was first discovered by expressing Yih1 at native levels and pulling it down from yeast whole cell extract (Sattlegger et al., 2004). Only monomeric actin was found as a Yih1 co-precipitation protein but not Gcn1. This indicated that the actin-Yih1 interaction is not dependent on Gcn1 *in vivo* (Sattlegger et al., 2004). This was supported by studies showing actin co-precipitation by Yih1 in strains deleted for *GCN1* (Sattlegger et al., 2011).

Actin forms part of the cytoskeleton and is one of the most abundant proteins inside the cell. It consists of filamentous structures called F-actin that are dynamically reorganised, depending on the cell's needs, using the monomeric actin (G-actin) as building blocks (Oda et al., 2009). Actin has a wide range of functions, from regulating cell division and cell motility to the intracellular transport of proteins and organelles and more (Dillon and Goda, 2005; Oda et al., 2009). In mammals, actin is additionally involved in synaptic plasticity and consequently processes such as memory formation (Abraham and Williams, 2003; Dillon and Goda, 2005).

A.15 Actin binds the central and C-terminal domains of Yih1

In order to further elucidate the role the Yih1 domains play in actin binding, overexpressed full length Yih1 and Yih1 fragments were used in a co-precipitation assay, in a similar fashion as for Gcn1 above (Sattlegger et al., 2011). Fragments consisting of the RWD or the ancient domain showed little actin binding. In fact, the RWD domain appeared to restrict actin binding because full length Yih1 showed a lower level of actin binding than a fragment containing the linker and the

C-terminus. The region in Yih1 that showed the strongest actin interaction ranges from amino acids 68 to 171, indicating that the central part is necessary for actin binding. Therefore, the actin and Gcn1 binding areas in Yih1 partially overlap. This suggested that Yih1 can only bind to one of the two proteins at the same time (Sattlegger et al., 2011).

The result that showed that the linker was needed for Yih1-actin interaction was obtained using strains in which endogenous Yih1 was still present. Interestingly, in *YIH1* deletion strains the ancient domain was necessary for actin binding in addition to the central region. This might suggest that the endogenous Yih1 acts as a bridge between actin and the Yih1 fragment, suggesting Yih1 dimerisation (Sattlegger et al., 2004). This was supported by the appearance of Yih1 multimers when purified Yih1 was subjected to crosslinking chemicals that stabilise protein interactions; however, yeast two-hybrid experiments and size exclusion chromatography showed no dimerisation (Burr, 2012; Sattlegger et al., 2011). It remains unknown if Yih1 forms dimers and whether this is important for Gcn2 inhibition.

As discussed in section A.13 Yih1 with mutations in helix 2 showed increased Gcn1 binding (Sattlegger et al., 2011) (cf. Figure A6). These mutations were investigated for their effect on actin-Yih1 interaction (Sattlegger et al., 2011). Yih1*H2 showed increased actin binding compared to the wild type, just like for Gcn1. The increase in actin binding should reduce the ability of Yih1 to inhibit Gcn2 because Yih1-Gcn1 interaction is reduced; however, this was probably counterbalanced by its ability to decrease Gcn1-Gcn2 interaction with higher efficiency than the wild type (Sattlegger et al., 2011). Mutations in helix 3, on the other hand, did not affect actin binding. This suggested that only a part of the RWD domain is needed for actin binding.

These results suggested a model in which Yih1 is bound to actin and that this prevents Gcn1-Yih1 interaction (Figure A7) (Sattlegger et al., 2011). Assuming that actin and Gcn1 cannot bind Yih1 at the same time, actin binding to Yih1 would mask the Gcn1-binding site. Consequently, this would increase Gcn1-Gcn2 interaction and enable Gcn2 activation. However, it still needs to be determined if Gcn1 and actin can bind Yih1 at the same time. Even if this is the case it is possible that the actin-Yih1 interaction may restrict the ability of Yih1 to bind Gcn1. As mentioned above it was proposed that Yih1 might inhibit Gcn2 in certain

locations in the cell in order to allow a localised growth such as in the bud tip (Sattlegger et al., 2004). For this, Yih1 may be freed from actin and inhibit Gcn2, thus providing a way for the cytoskeleton to regulate Gcn2 activity (Sattlegger et al., 2011). However, it is not known what protein or molecule stimulates the release of Yih1 from actin.

A.16 Yih1-actin interaction may affect the GAAC

Actin is an essential protein (Giaever et al., 2002). Therefore, strains deleted for *ACT1* cannot be used to study if and how actin can influence the GAAC. There are two ways to approach this problem and they have been applied in previous research: One is the use of actin mutants and the other is to use diploid yeast strains where one actin allele was deleted.

A diploid yeast strain where one actin allele was deleted (*act1*Δ/*ACT1*) has been described as somewhat temperature sensitive (Wedman et al., 1992). Additionally, it was unable to grow at 37˚C when NaCl was added to the growth medium. Similarly, this strain showed a growth defect under amino acid starvation conditions caused by SM when grown at 37˚C, but not at 30˚C, and this is exacerbated by overexpressing Yih1 (Sattlegger et al., 2004). These results suggested the involvement of actin in the starvation response. Introduction of *YIH1* into an *act1*Δ/*ACT1 yih1*Δ/*yih1*Δ double mutant strain decreased growth on medium containing both SM and NaCl compared to introducing the vector control into the same strain (Sattlegger et al., 2004). Introduction of *ACT1* into the double mutant strain, however, resulted in a similar growth to strains that were only deleted for Yih1 (*ACT1*/*ACT1 yih1*Δ/*yih1*Δ strains). Although introduction of the vector had a negative effect on growth of the double mutant, the growth defect was stronger when *YIH1* was introduced. This suggested that a reduced amount of actin in an *act1*Δ/*ACT1* strain increased the amount of Yih1 that does not bind actin. This free Yih1 then was able to bind Gcn1 and inhibit the GAAC. However, these experiments were only done when NaCl was present and it needs to be verified if this is the case on medium containing only SM. Nonetheless, these results are consistent with the idea that there is a connection between Gcn2 and the cytoskeleton and that Yih1 may act as a bridge.

Another approach to test the connection of actin to the GAAC is to use a set of haploid actin mutants that were viable but showed impaired growth on rich or NaCl-containing media or exhibited sporulation defects (Whitacre et al., 2001). Use of these mutants could help us map the Yih1-binding site in actin. If actin is involved in the GAAC and Yih1 binds actin then actin mutant strains that disrupt this interaction should show a growth defect under amino acid starvation conditions because Yih1 is free to inhibit Gcn2. Twenty-four viable actin mutants were studied and five of them showed an impaired starvation response when grown on medium containing either SM or 3AT (Dautel 2012). If Yih1 is removed in these strains then wild type growth should be restored; however, this was not the case. Furthermore, if Yih1 is overexpressed in actin mutant strains that show reduced Yih1 binding then overexpression of Yih1 should result in a reduced binding to actin mutants compared to the wild type actin. However, most actin mutants showed an increased level of Yih1 binding (Dautel 2012). This was in part likely due to the overexpression of Yih1 which would drive an interaction although this does not explain why there was no increased binding of Yih1 to wild type actin. Thus, it was not clear from these experiments if Yih1 can be a mediator between actin and the starvation response.

Since Yih1 binds actin it was possible that removing Yih1 could affect cell structure (Sattlegger et al., 2004). In order to test this idea, yeast cells were stained with the F-actin binding rhodamine-phalloidin (Lengsfeld et al., 1974). Cells without Yih1 show less staining than cells with Yih1 but this did not correlate with any obvious morphological defects or changes in actin localisation inside the cell between *yih1*Δ and *YIH1*$^{+}$ strains. This suggested that Yih1 does not regulate the structure of the cell skeleton under these conditions. However, these results do not exclude the possibility that the cell skeleton regulates Yih1 and the GAAC on other levels or under different conditions.

A.17 Yih1 associates with ribosomes

As discussed earlier, Gcn1 and Gcn2 can bind ribosomes. One recent study presented evidence that Yih1 can also associate with the ribosome (Waller et al., 2012).

One approach to verify if there is a Yih1-ribosome association is to use co-sedimentation assays, in a similar fashion as was done for the Gcn1- and Gcn2-ribosome association discussed earlier. If Yih1 can bind to translating ribosomes then one would expect to find Yih1 co-sedimenting with the polysome-containing fractions. This is indeed the case: In an assay where overexpressed Yih1 was used cellular Yih1 was found together with polysomes (Waller et al., 2012). Similar results were found with natively expressed Yih1. Interestingly, Yih1 appeared to bind to the ribosome under both starvation and non-starvation conditions, as shown by co-sedimentation assays (Waller et al., 2012). This suggested that starvation does not affect Yih1-ribosome interaction.

Another approach was to use overexpressed Yih1 in order to investigate if it can co-precipitate ribosomal subunits. The two ribosomal proteins RPS22 and RPL39 were found in a complex specifically with Yih1 (Waller et al., 2012). This result was not reproduced with native Yih1, presumably due to its low expression levels. Since Yih1 binds Gcn1, and Gcn1 is a known ribosome-binding protein, it was possible that Gcn1 acts as a bridge between Yih1 and the ribosome. To test this, the same co-purification experiment was repeated using a strain lacking Gcn1. The Yih1-ribosome complex formation remained intact. This showed that Yih1 can associate with ribosomes and that this interaction is not dependent on Gcn1. However, this does not exclude the possibility that other, unidentified Yih1-binding proteins are involved.

The co-sedimentation assay was repeated using the Yih1 fragments that were used to study its interactions with Gcn1 and actin (Waller et al., 2012). Yih1 fragments consisting of the central domain alone or the central plus the C-terminal domain showed the highest level of polysome co-sedimentation. The N-terminal region (1-67 amino acids) of Yih1 showed a reduced co-sedimentation with polysomes compared to full-length Yih1, suggesting that the N-terminal region restricts ribosome binding, similar to the interaction of this fragment with actin. In addition, the C-terminal region does not restrict Yih1-ribosome interaction, in contrast to Gcn1 (Figure A6).

A.18 Gcn1, actin and ribosomes overlap in their binding regions in Yih1

To summarise the findings of the studies using Yih1 fragments, the Yih1 linker region as well as parts of the RWD and ancient domain were sufficient for binding to Gcn1 and the ribosome (Figure A6) (Sattlegger et al., 2011). The same fragments are sufficient for an interaction with actin but only when native Yih1 is present in addition to the fragments. If no Yih1 is present then the full ancient domain is required for actin binding. While Gcn1, actin and ribosomes overlap in their binding regions in Yih1 each protein likely uses different binding determinants in Yih1 (Waller et al., 2012).

A.19 Actin may promote Gcn2 activity by inhibiting Yih1

The following regulatory model for Yih1, Gcn1, Gcn2 and actin was proposed (Sattlegger et al., 2004; Waller et al., 2012) (Figure A7): It was found that Gcn1 and Gcn2 are bound to the ribosome which enables them to sense the starvation signal in the form of uncharged tRNA (Marton et al., 1997; Ramirez et al., 1991). Yih1 presumably can change its interaction partner between Gcn1, actin and ribosomes (and likely other, yet to be identified proteins). It could perform its regulatory function depending on the cellular need and in a rapid fashion due to the close proximity to Gcn1 in order to respond to an as yet unknown signal. Of note is that research already showed an association between protein synthesis and the cytoskeleton, supporting the idea that Yih1 may be involved in the cross-communication between the two entities (Chatterjee et al., 2006; Dias et al., 2008; Valouev et al., 2002; Zanelli and Valentini, 2005). When Yih1 is bound to G-actin then Yih1 may be inactive and Gcn1 and Gcn2 are able to interact. Conversely, if Yih1 is freed from G-actin then Yih1 may be able to bind Gcn1 and subsequently inhibit Gcn2. However, we do not know what signal or condition breaks the Yih1-actin interaction or brings them together. One possibility is that Yih1 is displaced by other actin-binding proteins or other Yih1-binding proteins (Sattlegger et al., 2004).

A.20 IMPACT, the mammalian Yih1 homolog, inhibits Gcn2 activity and binds actin and ribosomes

IMPACT is the mammalian homolog of Yih1. Just like Yih1 in yeast, mouse IMPACT (mIMPACT) acts as an inhibitor of mouse Gcn2, leading to low levels of the transcription activator ATF4 (the mammalian functional homolog to yeast Gcn4) (Cambiaghi et al., 2014; Pereira et al., 2005). Furthermore, the overexpression of mIMPACT in yeast cells decreases eIF2α phosphorylation by Gcn2, similar to the effects of Yih1 overexpression. This suggests a similar role for IMPACT in mammals to that of Yih1 in yeast: Inhibition of Gcn2 to ensure optimal levels of protein synthesis. Supporting this, IMPACT was able to precipitate with both mouse and yeast Gcn1 in a pulldown, indicating that IMPACT can disrupt Gcn1-Gcn2 interaction and thus Gcn2 activity (Pereira et al., 2005). In addition, it is possible that IMPACT also binds to and is regulated by actin. When IMPACT was expressed in yeast *YIH1* deletion strains it was indeed in a complex with actin (Waller et al., 2012). In a similar fashion to Yih1, mIMPACT could regulate Gcn2 in a defined spatial and temporal manner and would be itself regulated by other proteins. In fact, IMPACT also associates with ribosomes, providing further evidence for the functional conservation between Yih1 and IMPACT (Roffé et al., 2013).

A.21 Additional Yih1-binding proteins (YBP) must exist

Interactions between proteins are essential for the functioning of a cell. In order to understand how the cell works we would need to know all the interactions between proteins (von Mering et al., 2002). The function of Yih1 is just beginning to be understood; likewise, little is known about how Yih1 is regulated. Yih1 may be an important player in regulation of the starvation response due to its ability to inhibit Gcn2. Therefore, cells need to ensure a fine-tuned regulation of Yih1 itself in order to survive. To date, Gcn1 and actin are confirmed Yih1-binding proteins and, as discussed above, Gcn1 is involved in Gcn2 activation. The search for additional YBP that forms the core of this thesis is based on the idea that Gcn1

and actin are unlikely to be the only YBP, as justified by the following observations:

A number of large-scale yeast protein-protein interaction studies exist (Babu et al., 2012; Costanzo et al., 2010; Gavin et al., 2006; Ho et al., 2002; Ito et al., 2001; Krogan et al., 2006; Peng et al., 2003; Tarassov et al., 2008; Uetz et al., 2000; Yu et al., 2008). Each study tested hundreds or thousands of proteins to find their interaction partners. Thus, one would expect that they identified Gcn1 and actin as Yih1-binding proteins. However, this was not the case and only one of several yeast large-scale studies identified Gcn1 as a co-precipitator of Yih1 and none identified actin (Tarassov et al., 2008). One explanation for this discrepancy is that not all protein-protein interactions were identified (Collins et al., 2007; Yu et al., 2008). For example, high-throughput studies are less likely to identify weak or transient interactions as they can be lost during the washing steps needed to remove unspecific interactions (Perkins et al., 2010). Stabilisation of protein interactions by crosslinking is a way to retain weak or transient interactions; however, so far this was only attempted at a small scale (Tagwerker et al., 2006). Some studies used mass spectrometry for protein identification which is biased against low abundant or small proteins. This was likely because the number of peptides that was available for identification was not significant (Gavin et al., 2006; Krogan et al., 2006). In addition, large scale studies contain false negatives (interactions that exist *in vivo* but were not identified) and false positives (interactions that were identified but that do not occur *in vivo* or that were not reproducible in an independent system) (Brückner et al., 2009). Thus, in order to confirm an interaction it needs to be validated by, for example, co-precipitation assays *in vivo* and *in vitro*, as used to identify the YBP Gcn1 and actin (Brückner et al., 2009). Taken together, this supports the idea that not all YBP are known yet.

Further evidence for additional YBP comes from structural modelling: Several conserved amino acid residues exist in the ancient domain of Yih1 (Sattlegger et al., 2011). Specifically, the ancient domain of both pro- and eukaryotes contains four sequence motifs in the loop regions between β-sheets B1 and B2, α-helix H1 and B3, B4 and H2, and B5 and H3. The conservation of these amino acid residues indicated the presence of important features in this domain that are needed for protein and in turn cellular function (Caffrey et al., 2004; Sattlegger et al., 2011). The observation that these motifs are clustered on the

same side of the molecule and are exposed to the solvent indicated that they are forming a protein binding surface (Sattlegger et al., 2011). As discussed above the Gcn1-binding region in Yih1 is located in the linker and in part of the RWD domain while the ribosome binds the linker and parts of the RWD and ancient domains (cf. Figure A6). Actin binding to Yih1 may require the linker and most or part of the ancient domain. However, most of the conserved motifs are outside of the areas that are needed for Gcn1, actin or ribosome binding. Taken together, this suggests the existence of unidentified YBP which use the ancient domain to dock to Yih1.

FoldIndex is a computational tool that can be used to predict structured and unstructured regions in proteins (Prilusky et al., 2005). For Yih1 this revealed that the central region (the linker) is mostly unstructured (unfolded) and flexible (Sattlegger et al., 2011). An unstructured linker, together with the structured RWD and ancient domain predicted by modelling, is in agreement with circular dichroism experiments that indicated that almost 30% of Yih1 consists of random structure (Sattlegger et al., 2011). It was predicted that regulatory proteins are more likely to contain unstructured regions, in concordance with the idea that Yih1 is a regulatory protein (Iakoucheva et al., 2002). It is known that flexibility allows for a high specificity and high reversibility of interactions and this permits a plastic response to different stimuli (Dyson and Wright, 2005). It was found that a protein containing a linker remains relatively flexible but assumes a rigid structure which is triggered upon interaction with its target protein or DNA (Laity et al., 2000; Young et al., 2001). Therefore, this so-called “snap lock” mechanism would increase binding affinity when a flexible protein region is involved. For Yih1 it is possible that it interacts with some of its binding partners by “snapping” its (structured) N- and C-terminal domains into place (Sattlegger et al., 2011; Waller et al., 2012). In other words, the RWD or ancient domain would be restricted in their interactions unless the linker assumes a certain conformation. As this conformation change is induced by other factors this then implies that other YBP exists. However, so far, no such proteins are known. It is also possible that Gcn1 or actin induces these changes but this not known.

Yih1 was found as a potential novel interaction partner of Cdc28, a cell cycle regulator (Dautel, 2012). This interaction was not identified in the large-scale studies, possibly due to false negatives, and provided further evidence for the incompleteness of the Yih1 interactome. Cdc28 plays a role in bud emergence by

activating other proteins involved in bud growth (Enserink and Kolodner, 2010). It was proposed that Yih1 inhibits Gcn2 in the bud tip in order to allow protein synthesis and cell growth (Sattlegger et al., 2011). Thus, it is possible that Yih1 is activated by Cdc28 and that this regulates Gcn2 activity in the bud tip (Dautel, 2012). However, Yih1 has not been identified as a target of Cdc28 (Enserink and Kolodner, 2010; Ubersax et al., 2003). This may be because not all Cdc28 targets have been identified yet or could suggest the existence of a YBP that acts as a bridge between Yih1 and Cdc28.

A.22 Hypothesis and objectives

Yih1 is negative regulator of the GAAC as it restricts Gcn2 activation but how Yih1 is regulated is not well understood. While Gcn1 and actin are confirmed interaction partners, it appears unlikely that they are the only two players in the Yih1-mediated starvation response inhibition, as outlined in section A.21. Therefore, the following hypotheses were formulated:

> **Hypothesis 1**: Additional proteins exist that bind to or are in a complex with Yih1.
>
> **Hypothesis 2:** Additional proteins exist that bind to or are in a complex with Yih1 and they promote Gcn2 activation.

In order to test these hypotheses and in order to further our understanding of how Yih1 and Gcn2 are regulated the objectives in this work were as follows:

Objective 1: Identify proteins in complex with Yih1

In order to identify Yih1-binding proteins (YBP) two approaches will be used. In one approach, published large-scale yeast protein-protein interaction studies will be mined for proteins that are potentially in a complex with Yih1. In another approach, in-house co-precipitation experiments that will enhance the probability of finding weak or transient Yih1 interaction partners will be performed, whether by driving interactions by Yih1 overexpression or by stabilising interactions with crosslinking chemicals.

Data from both approaches will be compared and compiled into a list of all proteins found to be in complex with Yih1, the draft Yih1 interactome.

Objective 2: Identify YBP required for promoting Gcn2 activity

The next objective is to screen for novel YBP that promote Gcn2 activation. For this, yeast strains deleted for one YBP at a time are used. These deletion strains will be investigated for their ability to overcome amino acid starvation conditions and how this affects Gcn2 activity. If a protein is needed to promote Gcn2 activity then removing it from the cell should reduce Gcn2 activity.

In order to simplify the nomenclature the term “Yih1-binding protein(s)” (YBP) from here on can refer to proteins that bind directly to Yih1 or to proteins that bind indirectly via a directly interacting YBP (Figure A8).

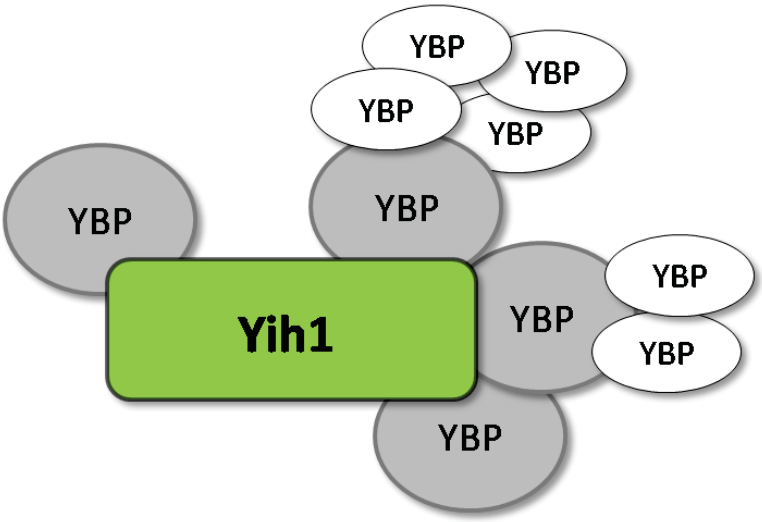


Figure A8: Yih1-binding proteins (YBP) in this study refers to proteins that are in a complex with Yih1 either directly (*larger grey ovals*) or indirectly (*smaller white ovals*).

Chapter B

Material and methods

Chapter B: Materials and methods

B.1 Media

Media for yeast and bacterial cultures were prepared using MilliQ® water and sterilised at 121˚C for 20 min. For yeast media carbon sources and amino acids were sterilised separately from the media and added prior to use. Liquid media were cooled to and stored at room temperature and supplements were added prior to use. Solid medium was prepared by adding agar to the medium at a final concentration of 2% (w/v). Solid medium was cooled down and supplements were added before solidification and then poured into petri dishes. Plates were stored at 4˚C or at room temperature for YPD plates.

All chemicals and salts were purchased from Sigma-Aldrich, Thermo Fisher, Invitrogen, Formedium, Ajax Finechem, BioRad, Roche, or otherwise mentioned.

Bacterial medium
LB medium 1% (w/v) tryptone (Formedium) 0.5% (w/v) NaCl (Ajax) 0.5% (w/v) yeast extract (Formedium)

Yeast media
Yeast extract peptone dextrose (YPD) (rich medium) 1% (w/v) yeast extract (Formedium) 2% (w/v) bacto-peptone (Formedium) 2% (w/v) glucose (Sigma-Aldrich)
Yeast extract peptone glycerol (YPG) 1% (w/v) yeast extract (Formedium) 2% (w/v) bacto-peptone (Formedium) 3% (v/v) glycerol (Sigma-Aldrich)
Synthetic dextrose (SD) (minimal medium) 0.145% (w/v) yeast nitrogen base without amino acids (Formedium) 0.5% (w/v) ammonium sulfate (Ajax) 2% (w/v) glucose (Sigma-Aldrich) or 2% (w/v) galactose (Formedium)

Table B1: Medium supplements

	Solvent	Final concentration
Antibiotics		
Ampicillin	water	100 µg/mL
Kanamycin	water	50 µg/mL
G418	water	200 µg/mL
Drugs		
Sulfometuron methyl (SM)	DMSO	1-4 µg SM/mL DMSO
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	water	10 mM
Amino acids		
Glutamate	water	10.0 mg/mL
Histidine	water	21.0 mg/mL
Isoleucine	water	6.6 mg/mL
Leucine	water	13.0 mg/mL
Lysine	water	7.3 mg/mL
Methionine	water	7.5 mg/mL
Tryptophan	water	8.0 mg/mL
Uracil	water	2.2 mg/mL
Valine	water	2.9 mg/mL

B.2 Growth conditions

Bacterial growth conditions

Escherichia coli cultures were grown at 37˚C in LB liquid or on LB agar medium supplement with appropriate antibiotic (Table B1) or without. When grown in liquid medium cultures were shaken at 160 rpm.

Yeast growth conditions

Saccharomyces cerevisiae cultures were grown at 30˚C or at the semi-permissive temperatures of 14˚C and 37˚C. Cultures were grown in liquid or on solid SD medium supplemented with appropriate amino acids or on/in YPD or YPG. When grown in liquid medium cultures were shaken at 160 rpm.

Storage of bacterial and yeast strains

Bacterial cultures were stored permanently at -80˚C in 66% (v/v) glycerol. Yeast cultures were stored permanently at -80˚C in 30% (v/v) glycerol.

B.3 DNA isolation and purification

Commercial plasmid isolation kits

A commercially available plasmid isolation kit was used when highly clean DNA was required, for example for sequencing reactions. Kits were purchased from either Invitrogen or Qiagen and the plasmid extraction was done according to the manufacturers' protocol.

Modified mini alkaline-lysis precipitation procedure

The following is modified from the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit, The Perkin-Elmer Corporation. Bacterial cultures were grown overnight at 37˚C in 5 mL of LB with an appropriate amount of antibiotic. The cultures were pelleted in 1.5 mL aliquots of culture for 1 min in a microcentrifuge at 13k rpm and at 4˚C. The supernatant was removed by aspiration and the pellet was resuspended in 200 µL of GTE buffer by pipetting up and down. 300 µL of 0.2 M NaOH / 1% SDS were added and the contents of the tube were mixed by inversion until the solution clears and then incubated on ice for 5 min. The solution was neutralised by adding 300 µL of 3.0 M potassium acetate (pH 4.8), mixed by inverting the tube and incubated on ice for 5 min. After centrifuging for 10 min at 13k rpm and at room temperature the supernatant was transferred to a clean tube. 1 µL of RNase A (DNase-free, Roche, 500 µg/mL) was added and incubated at 37˚C for 10-20 min.

After the RNase A treatment the supernatant was extracted twice with 400 µL of chloroform. The layers were mixed by hand for 30 s after each extraction. At each extraction step the tube was centrifuged for 1 min to separate the phases and the aqueous phase was transferred to a clean tube. The total DNA was precipitated by adding an equal volume of 100% isopropanol and immediately centrifuging the tube for 10 min at 13k rpm and at room temperature. The DNA pellet was washed with 500 µL of 70% ethanol, centrifuged for 5 min at 13k rpm and at room temperature and then dried under vacuum for 3 min or until the pellet was dry. The pellet was dissolved in 32 µL of deionised H_2O.

GTE Buffer
50 mM glucose
25 mM Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)

DNA quantification

The quantity of DNA was measured by agarose gel electrophoresis. Here the brightness of the band to be quantified was compared with the brightness of bands of known concentration.

Agarose gel electrophoresis

Agarose gels of desired concentrations (0.8-2% w/v) were prepared in 1X Tris-acetate-EDTA (TAE) buffer containing 1 µL/mL ethidium bromide. Samples were mixed with 6X DNA loading dye and separated on the agarose gel at a constant voltage of 80V using the BioRad PowerPac 3000. The DNA was visualised on an UV transilluminator and images of the gel were taken using the Gel Doc Imager (BioRad).

TAE buffer (50X)
2 M Tris
1 M acetate
100 mM EDTA
pH 8.1

DNA loading dye
0.25% bromophenol blue
0.25% xylene cyanol
50% glycerol

B.4 Transformation of *E. coli*

Preparation of competent cells using the calcium chloride method

A single *E. coli* DH5α colony was incubated to 5 mL of LB medium without antibiotic and incubated overnight at 37˚C. 400 µL of this saturated cultures was inoculated into 40 mL LB without antibiotic and grown to OD_{600nm} = 0.6 at 37°C (3-4 h). Cells were harvested by centrifugation at 3000 rpm for 10 min and 4°C. The pellet was resuspended in 20 mL $CaCl_2$ solution and incubated on ice for 30 min. After centrifugation at 3000 rpm for 10 min and 4˚C, the pellet was resuspended in 4 mL ice cold glycerol/$CaCl_2$ solution and left on ice for 4 to 21 h. Aliquots of 200 µL were stored at -80°C.

Calcium chloride solution
50 mM $CaCl_2$
Sterile filtered and stored at 4°C

Glycerol/$CaCl_2$ solution
15% (v/v) glycerol
50 mM $CaCl_2$
Sterile filtered and stored at 4°C

Transformation of bacteria using the heat shock method

Competent DH5α cells were aliquoted into a pre-chilled 1.5 ml tube. 1-5 µL of plasmid DNA were added (up to 5% of cell volume) to 100 µL of competent cells and incubated on ice for 10 minutes. The cells were heat shocked the cells at 42°C for 1 min and 1 mL of LB medium was added. The cells were shaken at 160 rpm at 37˚C for 1 hour and then plated on solid LB medium containing appropriate antibiotics and grown at 37˚C overnight.

B.5 Transformation of yeast using the lithium acetate method

Preparation of competent yeast

50 mL of YPD medium was inoculated with 1-2 mL of a saturated overnight culture and grown at 30˚C at 160 rpm until OD_{600nm} = 0.6. Next the cells were centrifuged at 4000 rpm for 3 min at 4˚C and resuspended in 500 µL of Solution 1. These cells were incubated at 30˚C for 1 h and used immediately or stored overnight at 4˚C for use the next day.

Solution 1
10 mM Tris-HCl
1 mM EDTA
100 mM LiOAc (lithium acetate)

Transformation of yeast

Herring sperm ssDNA (10 mg/mL) was boiled for 10 min and then cooled on ice. 100 µL of competent cells were combined with 5 µL of plasmid DNA and 5-10 µL of herring sperm DNA and incubated at 30˚C for 15 min. Next, 700 µL of Solution 2 were added and incubated for 1.5 h at 30˚C while shaking. Afterwards, cells were heat shocked at 42˚C for 15 min and then left on ice for 2~5 min. Cells were pelleted at 1000 rpm for 3 min and the remaining supernatant removed. The pellet was resuspended in about 100-200 µL SD and then transferred to solid

medium containing appropriate supplements. Cells were incubated at 30 ˚C for 2~4 days or until colonies were visible.

Solution 2
10 mM Tris-HCl
1 mM EDTA
100 mM LiOAc
40% PEG-3500

B.6 Preparation of yeast whole cell extract

Cells were grown and harvested as outlined in the individual sections below. Cells were broken either physically or chemically.

Physical breakage

Cell cultures were harvested and then transferred to round bottom tubes (Visweswaraiah et al., 2011a). Cell pellets were resuspended in 100-200 µL of breaking buffer and 700 µL of glass beads were added. The cells were vortexed at maximum velocity for 30 sec then kept on ice for 30 sec. These steps were repeated 10 times for each sample. Afterwards, cells were centrifuged at 13k rpm for 10 min and at 4 °C and the supernatant was transferred to a new tube. The pellets were either used directly or stored at -80˚C.

Breaking buffer (50 mL)
30 mM HEPES-KOH (pH 7.5)
50 mM KCl
10% (v/v) glycerol
1 mM PMSF
1 mM DTT
1 complete inhibitor tablet without EDTA

Alkaline lysis

A 2 mL overnight culture was pelleted for 4 min at 4000 rpm and resuspended in 200 µL 0.1 M NaOH (protocol adapted from Kushnirov 2000). After incubation for 5 min at room temperature cells were pelleted again and resuspended in 50 µL 2X SDS-PAGE loading buffer (see section “SDS-PAGE” below). Finally, samples were heated at 80-90˚C for 10 min to break cells.

B.7 Estimation of protein concentrations using the Bradford method

This protocol is based on Bradford (1976). Bovine serum albumin (BSA) standards of increasing protein concentrations (2 µg/µL, 4 µg/µL, 6 µg/µL, 8 µg, 10 µg/µL, 12 µg/µL, and 14 µg/µL) were made by diluting a stock of 10 mg/mL. 1 µL of whole cell extract with an unknown protein concentration were taken in duplicate in a 96 well clear microtiter plate. To each samples 200 µL Bradford solution was added and the mixture was incubated for 5 min at room temperature. The absorbance was read at 595 nm in a FLUOstar OPTIMA plate reader (BMG Labtech). A standard curve was plotted with the absorbance against protein concentration of the known standards. The unknown protein concentration of the samples was determined by comparison of its absorbance with the standard curve.

Bradford reagent
0.5 mg/mL Coomassie Blue G250
25% methanol
42.5% H_3PO_4
0.05 M NaOH
Stored in dark bottle

B.8 Formaldehyde-crosslinked whole cell extract

Yeast overnight cultures were inoculated into 300 mL minimal (SD) medium containing appropriate supplement (Sattlegger and Hinnebusch, 2005). The cells were grown at 30˚C at 140-160 rpm to exponential phase (OD_{600nm} = 1). 75 g shaved ice and 0.1 to 1% formaldehyde (final concentration in the ice-formaldehyde mixture) were added to a 500 mL centrifuge bottle. The yeast culture was added and kept on ice for 1 h or for 10 min at room temperature or at 4˚C. If kept for 1 h they were shaken every 15 min. 2.5 M glycine (0.1 M final) was added to quench the crosslinking reaction and then the cells were harvested by centrifugation at 4,200 rpm for 5 min and at 4˚C. The cells were transferred to

round bottom tubes and physical cell breakage was done as described above in section B.6.

B.9 SDS-PAGE

Protein samples were separated using a gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 1% (w/v) agarose in 1.5 M Tris-HCl (pH 8.8) was used to seal the gaps between the spacers in between two glass plates. 20 mL of 4% premix was added into one chamber of the gradient mixer and 20 mL of 17% premix was added to the other chamber. 20 µL of TEMED (N,N,N',N'-Tetramethylethylenediamine) and 200 µL of 10% APS (ammonium persulfate) was added to each chamber and the contents were mixed using magnetic stirrers. The valve between the chambers was opened, mixing the two premixes and the resulting acrylamide gradient was allowed to flow between the glass plates. A comb was added between the glass plates to form wells for samples loading. The gel was allowed to solidify for at least 30 min. The resulting gel had the dimension 17 cm x 14.5 cm x 1.8 mm (height, width, thickness). The solid gel was mounted into the gel electrophoresis unit and covered with running buffer. Samples were resuspended in loading buffer, heated at 80-90˚C for 10 min and loaded into the wells of the gel. The gel was run at 250V and 100 mA until the blue dye front reached the end of the gel.

Premixes	4%	17%
ddH2O	75.3	48.8
Acrylamide 40%, 29:1	12	68
Tris-HCl (pH 8.8)	30	40
10% SDS	1.2	1.6

Running buffer
25 mM Tris
192 mM glycine
1% SDS (w/v)

2X SDS-PAGE loading buffer
0.125 M Tris-HCl (pH 6.8)
4% (w/v) SDS
20% glycerol
0.1% (w/v) bromophenol blue
10% (v/v) beta-mercaptoethanol

B.10 Coomassie blue staining proteins in acrylamide gels

The gel was covered with staining solution and left to shake overnight at room temperature. The staining solution was removed and the destaining solution was added, shaken and removed. Adding and removing destaining solution was repeated until protein bands are seen without background staining of the gel.

Staining solution
0.1% (w/v) Coomassie blue R250
20% (v/v) methanol
10% (v/v/) acetic acid

Destaining solution
50% (v/v) methanol
10% (v/v) acetic acid

B.11 Western blot

Protein transfer

For western blotting the proteins were transferred from the gel onto PVDF membranes (pore size 0.45 µm, Thermo Fisher). The PVDF membrane was soaked in methanol for 10 sec and equilibrated in transfer buffer before usage. The gel and membrane were put between two layers of Whatman paper (Thermo Fisher) and immersed in transfer buffer. The transfer was run for 3 h at 24 V and 1 A.

Transfer buffer
25 mM Tris
192 mM glycine
20% (v/v) methanol

Ponceau S staining of PVDF membrane

To visualise the general protein contents on the PVDF membrane it was submerged in 0.8% Ponceau S (in 1% (v/v) acetic acid) after the transfer and shaken for 10-15 min. Liquid was removed and washed with 1% acetic acid to remove background and until protein bands were visible. The protein staining was removed by washing with TBS-T several times. Alternatively, TBS-T was added after removing Ponceau S and left at 4˚C overnight. TBS-T was removed and the membrane was washed with 1% acetic acid.

Immunological detection of proteins

To visualise specific proteins on the membrane it was first submerged in 5% (w/v) milk (Pams) at room temperature for 30 min. The membrane was incubated for 1 h with an appropriate primary antibody (Table B2). Antibodies were diluted either in 5% milk or in 3% BSA. The membrane was washed with TBS-T three times for 5, 10, and 15 min and then it was incubated with a secondary antibody with a horse radish conjugate (Table B2) for 1 h. Next, the membrane was washed again three times. For the detection of the specific protein signal the membrane was incubated with Super Signal West Pico Chemiluminescence solution (Thermo Fisher) or with equal volumes of Solution A and B. Without removing the liquid the chemiluminescence signal on the membrane was detected with the luminescent Image Analyser LAS-4000 (Fujifilm). Densitometric analysis of western blot signal was done using the open source software ImageJ (http://imagej.nih.gov/ij/).

Solution A	Solution B
100 mM Tris pH 8.5	100 mM Tris (pH 8.5)
2.5 mM luminol	0.02% H_2O_2
0.4 mM p-coumaric acid	

Table B2: Antibodies used in this study. mc/pc: mono-/polyclonal (if known).

Primary antibody	Dilution	Secondary antibody	Source
Actin	1:1000	mouse	Chemicon
Actin (pc)	1:1000	rabbit	Santa Cruz
Cdc28 (pc)	1:1000	goat	Santa Cruz
eEF1A	1:1000	rabbit	Terri Kinzy
eEF3A	1:5000	rabbit	Terri Kinzy
eIF2α-P (pc)	1:5000	rabbit	Invitrogen
FLAG	1:1000	mouse	Sigma-Aldrich
Gcd6	1:1000	rabbit	Alan Hinnebusch
Gcn1 (HL1405)	1:1000	rabbit	Vazquez de Aldana (1995)
Gcn2	1:1000	guinea pig	Beatriz Castilho
Gcn20 (CV1317)	1:1000	rabbit	Vazquez de Aldana (1995)
GST (pc)	1:1000 to 2000	rabbit	Santa Cruz
His (pc)	1:200 to 1000	rabbit	Santa Cruz
His	1:200 to 1000	mouse	Abcam
Myc (mc)	1:500	mouse	Roche
Pgk1 (mc)	1:1000 to 5000	mouse	Invitrogen
Yih1	1:1000	rabbit	Beatriz Castilho

Table B2 (cont'd): Antibodies used in this study.

Secondary antibody	Dilution	Source
rabbit	1:100,000	Pierce
mouse	1:50,000	Pierce
guinea pig	1:5000	Santa Cruz
goat	1:5000	Pierce

Parts of the experiments shown in Chapter D.3 were done by Mathias Joachim who was supervised by Michael Bolech.

B.12 Protein-protein interaction assays *in vitro*

Growing and breaking of cells

E. coli strains harbouring a plasmid containing His_6-Yih1 or GST-Gcn1[2052-2428] were grown in 300 mL LB medium with ampicillin or kanamycin, respectively, until OD_{600nm} = 0.8. The expression of proteins was induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) and cultures were transferred to room temperature for 3 h. Cells were harvested by centrifuging at 13,000 rpm for 5 min and at 4˚C. The pellet was resuspended in 1 mL of breaking buffer. 20 µL of lysozyme (10 mg/mL) was added to break cells and the solution was incubated for 30 min at 4˚C. The cell extract was further treated with 1 µL of RNase (20 mg/mL) and 16 µL DNase (10 U/µL) (Roche) at 4˚C until liquid and subsequently centrifuged at 13,000 rpm for 5 min and at 4˚C. The supernatant containing the soluble protein fraction was used for the subsequent purification of His_6-Yih1 or GST-Gcn1[2052-2428].

Purification of proteins

For each sample, 60 µL [50% v/v] of Ni^{2+}-charged resin (BioRad Profinity iMac) or 12.5 µL [50% v/v] of glutathione beads (Thermo Fisher) was washed with breaking buffer. The supernatant containing His_6-Yih1 or GST-Gcn1[2052-2428] was added to the nickel or glutathione beads, respectively and incubated on a roller at 4˚C for 1 h. The nickel beads were washed with breaking buffer containing 5 mM imidazole. Proteins were eluted in two steps from the nickel beads with buffer containing 250 mM imidazole. Glutathione beads were washed with

breaking buffer three times and proteins were eluted in two steps with breaking buffer containing 10 mM reduced glutathione. 15 µL of each eluate was mixed with an equal amount of 2X loading dye and half of it was separated via SDS-PAGE. For formaldehyde crosslinking, equal volumes of samples containing His_6-Yih1 or GST-Gcn1[2052-2428] were mixed, 1% (final) formaldehyde was added and the crosslinking was done for 10 min at room temperature. This was followed by a GST pulldown (see below in section B.13).

B.13 Protein-protein interaction assays *in vivo*

Growing and breaking of cells

Yeast cultures containing GST or His fusion proteins were grown in 300 mL selective SD medium containing appropriate amino acids with galactose as the sole carbon source until OD_{600nm} = 1. Cells were harvested as described in section B.6 and crosslinked as described in section B.8.

Glutathione S-transferase mediated pulldown

To reduce background binding in the GST pulldown 0.5 to 2 mg of whole cell extract was pre-adsorbed with 10 µL of agarose (GE Healthcare) for 30 min at 4˚C. This was followed by centrifugation at 10,000 rpm for 10 min and the supernatant was incubated with 12.5 µL [66% v/v] of washed GST beads for 2 h at 4˚C. After centrifugation at 1000 rpm for 3 min at 4˚C the supernatant was removed and the beads were washed five times using breaking buffer. The beads were resuspended in 2X loading buffer, heated at 90˚C and 15 µL was separated via SDS-PAGE. In addition, an input control of 50-100 µg of protein was separated on the same gel.

Poly His pulldown

30 µL [50% v/v] of Ni^{2+} beads was used per sample and no pre-adsorption was done. The subsequent steps are the same as for GST pulldown.

B.14 Mass spectrometry

The following description of the procedure was taken from the protocol of the Centre for Protein Research, Otago University, where the mass spectrometry was performed. Whole SDS-PAGE gels were sent to the Centre for Protein Research where bands were excised.

"Sample preparation

Excised protein spots/bands were subjected to in-gel digestion with trypsin using a robotic workstation for automated protein digestion (DigestPro Msi, Intavis AG, Cologne, Germany). The protocol for automated in-gel digestion is based on the method of Shevchenko et al., 1996. Eluted peptides were then dried using a centrifugal concentrator. A protocol for manual tryptic digestion can be downloaded at http://www.biochem.otago.ac.nz/cpr/protocols.html.

LC-MS/MS of tryptic peptides

Samples were re-solubilised in 5% [v/v] acetonitrile, 0.2% [v/v] formic acid in water and injected onto an Ultimate 3000 nano-flow uHPLC-System (Dionex Co, CA) that was in-line coupled to the nanospray source of a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, San Jose, CA). Peptides were separated on an in-house packed emitter-tip column (75 um ID PicoTip fused silica tubing (New Objectives, Woburn, MA) packed with C-18 material on a length of 8-9 cm) by a gradient developed from 5% [v/v] acetonitrile, 0.2% [v/v] formic acid to 80% [v/v] acetonitrile, 0.2% [v/v] formic acid in water at a flow rate of 200-500 nl/min.

Typical Instrument Setting for the LTQ-Orbitrap

Full MS in a mass range between m/z 300-2000 was performed in the Orbitrap mass analyser with a resolution of 60,000 at m/z 400 and an AGC target of 5e5. Preview mode for FTMS master scan was enabled to generate precursor mass lists. The strongest 5 signals were selected for CID (collision induced dissociation)-MS/MS in the LTQ ion trap at a normalised collision energy of 35% using an AGC target of 2e4 and one microscan. Dynamic exclusion was enabled with 2 repeat counts during 30 sec. and an exclusion period of 180 sec. Exclusion mass width was set to 0.01.

Data Analysis

For protein identification MS/MS data were searched against the SWISS-PROT amino acid sequence database (downloaded in May 2010) using the Mascot search engine (http://www.matrixscience.com). The search was set up for full tryptic peptides with a maximum of 3 missed cleavage sites. Carboxyamidomethyl cysteine, oxidized methionine, pyroglutamate (E, Q) were included as variable modifications. The precursor mass tolerance threshold was 10 ppm and the max. Fragment mass error 0.8 Da."

In addition to Mascot, the SEQUEST (http://fields.scripps.edu/sequest/) search engine was used by the Centre for Protein Research. The search results were filtered to remove any non-significant hits and all proteins listed in this work were found with a peptide false discovery rate (FDR, the percentage of the total number of identified peptide sequences for a protein that are incorrect) of below 1%. A significant identification from either of the two search engines was sufficient for concluding that a peptide was identified. The score is the cumulative protein score based on summing ion scores of the unique peptides identified for that protein.

B.15 Semi-quantitative growth assays

Yeast were grown overnight in duplicate in 4 mL of appropriate liquid medium at 30˚C. The cultures were subjected to four 10-fold serial dilutions and 5 µL of each diluted and 5 µL of undiluted were transferred to the appropriate solid medium. Plates were incubated at various temperatures until colonies were visible and were regularly scanned using a document scanner to document their growth. The growth was scored using a 10 point system with "10" indicating that all in dilutions colonies were visible and "0" indicating that no colonies were visible.

Parts of the growth assays shown in Chapter D.2 were done by Mathias Joachim who was supervised by Michael Bolech.

B.16 Yeast sporulation and tetrad dissection

Diploids strains were formed by mixing haploid colonies of two different strains on solid YPD medium. The haploid *YIH1* deletion strain that was used, RRY42, was constructed and verified via PCR by Rashmi Ramesh. After incubation at 30˚C for one or two days colonies were picked with a toothpick, transferred to selective medium and incubated until colonies appear. Single cell colonies were transferred to YPD and allowed to grow one or two days at 30˚C. Colonies were transferred to sporulation medium and incubated at room temperature for 5-7 days. 5 µL of zymolyase was mixed with 45 µL of 1 M sorbitol and cells were added to this using a toothpick and incubated at 30˚C for 5 min. 150 µL of ice cold sterile water was added and the tube was kept on ice for 10 min. 10 µL of the sample was transferred to YPD medium and the tetrads were dissected using the MSM400 dissection microscope (Singer Instruments). Afterwards the plate was incubated at 30˚C for two or three days. Cells were then transferred to appropriate selection medium to test for the double deletion of genes (see Figure D11 and Appendix C).

Table B3: Yeast strains used in this study.

Strain	Genotype	Source
BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Giaever et al. 2002
BY4742	MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Giaever et al. 2002
BY4743	MATa/MATα *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 MET15/met15Δ lys2Δ0/LYS2*	Giaever et al. 2002
H1511	MATα ura3-52 trp1-63 leu2-3,112 GAL2+	Foiani 1991
H2556	H1511, and *GCN1* deletion	Vazquez de Aldana and Alan Hinnebusch, unpublished
H2557	H1511, and *GCN2* deletion	Vazquez de Aldana and Alan Hinnebusch, unpublished
H2558	H1511, and *GCN20* deletion	Vazquez de Aldana and Alan Hinnebusch, unpublished
ESY11001b	H1511, and *yih1*::kanR	Waller et al. 2012
TKY864	MATα *leu2-3,112 his4-713 ura3-52 trp1Δ tef2 Δ 2 tef1::LEU2 met2-1* pTKB731 (*TRP1* 2 μ *TEF1*)	Visweswaraiah et al. 2011
TKY865	MATα *leu2-3,112 his4-713 ura3-52 trp1Δ tef2 Δ 2 tef1::LEU2 met2-1* pTKB731 (*TRP1* 2 μ *TEF1-HIS6*)	Visweswaraiah et al. 2011
RRY42	BY4742, and *YIH1* deletion	Rashmi Ramesh and Evelyn Sattlegger (unpublished)
MBY77-2-11a	*hsc82*::KanMX4 (BY4741) and *yih1*::URA3 (BY4742)	This study
MBY77-3-4a	*tef4*::KanMX4 (BY4741) and *yih1*::URA3 (BY4742)	This study
MBY77-10	BY4741, and pRS315	This study
MBY77-11	77-2-11a, and pRR04a	This study
MBY77-12	77-2-11a, and pRS315	This study
MBY77-15	YMR186W, and pRS315	This study
MBY77-16	RRY42, and pRS315	This study
MBY77-17	YDR283C, and pRS315	This study
MBY77-18	YPL240C, and pRS315	This study

Table B3 (continued): Yeast strains used in this study.

Strain	Genotype	Source
YML008C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 erg6::kanMX4*	Giaever et al. 2002
YMR145C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 nde1::kanMX4*	Giaever et al. 2002
YOR375C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gdh1::kanMX4*	Giaever et al. 2002
YOL051W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gal11::kanMX4*	Giaever et al. 2002
YDR129C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sac6::kanMX4*	Giaever et al. 2002
YDR393W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 she9::kanMX4*	Giaever et al. 2002
YEL051W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vma8::kanMX4*	Giaever et al. 2002
YKL080W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vma5::kanMX4*	Giaever et al. 2002
YKL119C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vph2::kanMX4*	Giaever et al. 2002
YKL126W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ypk1::kanMX4*	Giaever et al. 2002
YOR136W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 idh2::kanMX4*	Giaever et al. 2002
YOR133W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 eft1::kanMX4*	Giaever et al. 2002
YOR201C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pet56::kanMX4*	Giaever et al. 2002
YPL002C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf8::kanMX4*	Giaever et al. 2002
YGL066W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sgf73::kanMX4*	Giaever et al. 2002
YNL229C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ure2::kanMX4*	Giaever et al. 2002
YNL236W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sin4::kanMX4*	Giaever et al. 2002
YKR026C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gcn3::kanMX4*	Giaever et al. 2002
YKR059W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 tif1::kanMX4*	Giaever et al. 2002
YPR049C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cvt9::kanMX4*	Giaever et al. 2002
YAL047C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spc72::kanMX4*	Giaever et al. 2002
YIL125W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 kgd1::kanMX4*	Giaever et al. 2002
YOL148C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spt20::kanMX4*	Giaever et al. 2002
YGL206C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 chc1::kanMX4*	Giaever et al. 2002
YJR090C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 grr1::kanMX4*	Giaever et al. 2002
YPL174C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 nip100::kanMX4*	Giaever et al. 2002
YJL130C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ura2::kanMX4*	Giaever et al. 2002
YOR198C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 bfr1::kanMX4*	Giaever et al. 2002
YKL138C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mrpl31::kanMX4*	Giaever et al. 2002
YPR036W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 vma13::kanMX4*	Giaever et al. 2002
YPL240C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hsp82::kanMX4*	Giaever et al. 2002
YMR186W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hsc82::kanMX4*	Giaever et al. 2002
YDR007W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 trp1::kanMX4*	Giaever et al. 2002
YGL195W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gcn1::kanMX4*	Giaever et al. 2002
YDR283C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gcn2::kanMX4*	Giaever et al. 2002
YCR059C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 yih1::kanMX4*	Giaever et al. 2002
YDR207C	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ume6*::kanMX4	Giaever et al. 2002
YDR245W	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mnn10*::kanMX4	Giaever et al. 2002

Table B4: Plasmids used in this study.

Plasmids	Gene	Selectable marker	Vector	Source
Bacterial gene fusions				
pES123_B1	*GST-GCN1[2052-2428]*	*AmpR*	pGEX-6p-3	Sattlegger et al. 2000
pES189_D1A	*HIS6-YIH1*	*KanR*	pET-28a	Sattlegger et al. 2004
pSL214-1a	*HIS6-YIH1* (2-258)-E87A,D90A [=*H2]	*AmpR*	pEMBLyex4	Su Jung Lee and Evelyn Sattlegger (unpublished)
Yeast gene fusions				
pES187-B1	*GST-YIH1* (2–258)	*AmpR URA3 leu2-d*	pES128-9	Sattlegger et al. 2004
pES247-8-1	*GST-YIH1* (68-258)	*URA3 leu2-d*	pES128-9	Sattlegger et al. 2011
pES330-5-3	*GST-YIH1* (2-258)-E87A,D90A	*AmpR URA3 leu2-d*	pES128-9	Sattlegger et al. 2011
pES196-6-4	*FLAG-YIH1*	*AmpR URA3 leu2-d*	pRS316	Sattlegger et al. 2004
pRS314	*TRP1*	*AmpR TRP1*		Sikorski et al. 1989
pRS315	*LEU2*	*AmpR LEU2*		Sikorski et al. 1989
pRS316	*URA3*	*AmpR URA3*		Sikorski et al. 1989
pES333-2-9	*MYC-YIH1*, low copy	*URA3*	pRS316	Waller et al. 2012
pSL203-1e	*HIS6-YIH1*	*AmpR*	pSL203-1e	Su Jung Lee and Evelyn Sattlegger (unpublished)
pSL214-1a	*HIS6-YIH1* (2-258)-E87A,D90A	*URA3 leu2-d*	pSL214-1a	Su Jung Lee and Evelyn Sattlegger (unpublished)
B4220	*trp1::kanMX3*	*AmpR KanR*	M3925	Voth et al. 2003
pEMBLyex4	empty vector	*AmpR URA3 leu2-d* 2 µ		Reed Wickner
pRR04a	*YIH1-VN*	*LEU2*	pRS315	Rashmi Ramesh and Evelyn Sattlegger (unpublishe
Tiling collection (Gene names in square brackets indicate partial gene sequences)				
YGPM26l19	*[YAL053W], OAF1, YAL049C, GEM1, YAL047W-A,* ***SPC72,*** *YAL046C, YAL045C, YAL044W-A, [GCV3], [PTA1]*	*KanR LEU2* 2 µ	pGP564	Jones et al. 2008
YGPM1b21	*[ORT1], tD(GUC)O, YOR131C, VPS17, EFT1, BAG7, YOR135C,* ***IDH2,*** *[SIA1]*	*KanR LEU2* 2 µ	pGP564	Jones et al. 2008
YGPM2a24	*[EFT1], BAG7, YOR135C,* ***IDH2,*** *SIA1, RUP1, YOR139C, SFL1, [ARP8]*	*KanR LEU2* 2 µ	pGP564	Jones et al. 2008

Chapter C

Identification of potential Yih1-binding proteins

Chapter C: Identification of potential Yih1-binding proteins

The first step in finding supporting evidence for the hypothesis that additional Yih1-binding proteins (YBP) exist and that they are involved in Gcn2 regulation is to identify proteins that potentially interact with or are in a complex with Yih1. This Yih1 interactome is based on two approaches: One uses data from already *published* large-scale protein-protein interactions screens and the other is based on the *in-house experiments*.

C.1 Assembly of the Yih1-interactome using published large-scale interaction screens

As outlined in the introduction only one large scale protein-protein interaction study identified the known Yih1-binding protein Gcn1 and none identified actin. This was one indication that not all YBP have been identified yet. Furthermore, because this data was based on large-scale experiments no characterisation of these putative YBP, for example on their influence on the starvation response, had been attempted. As a first step to find novel YBP the findings of the published interaction studies are discussed in more detail in this chapter. To date, ten large-scale protein-protein interaction studies exist for budding yeast (Table C1).

Table C1**:** Yeast large-scale protein-protein interaction studies and method used to purify YBP (in square brackets). YBP: Yih1-binding proteins. Y2H: yeast two-hybrid; PCA: protein-fragment complementation assay.

Identified YBP	Did not identify YBP
Krogan 2006 [affinity tag TAP]	Gavin 2002 [affinity tag TAP]
Uetz 2000 [Y2H]	Gavin 2006 [affinity tag TAP]
Ito 2001 [Y2H]	Ho 2002 [affinity tag FLAG]
Peng 2003 [affinity tag His]	Yu 2008 [Y2H]
Tarassov 2008 [PCA]	Babu 2012 [affinity tag TAP]

All ten studies used one of two types of approaches for finding physical protein-protein interactions. One is based on using tagged proteins to co-purify proteins that are in a complex with the tagged protein (Huang and Bader, 2009; Karagoz and Arga, 2013; Yu et al., 2008). The other approach is used to check if there is an interaction between two proteins and makes use of the yeast two-hybrid or protein complementation assays.

It is important to note that only five out of the ten studies identified Yih1 as a co-precipitator and potential Yih1-binding proteins (Table C1). Krogan and colleagues (2006) or Gavin et al. (2006) used an affinity tagging approach where tagged proteins are purified and co-precipitating proteins are identified (Gavin et al., 2006; Krogan et al., 2006). On the other hand, Uetz et al. (2000) and Ito et al. (2001) applied yeast two-hybrid assays. Tarassov et al. (2008) used a protein-fragment complementation assay.

Table C2**:** The number of potential Yih1-binding proteins (YBP) that were identified in each study. For affinity purification studies the number of additional proteins that co-purified with YBP is given. For Krogan 2006 the number of high scoring/low scoring YBP is given.

	Number of YBP	**Co-purified with YBP**
Krogan 2006	4/122	49/1,803
Uetz 2000	2	-
Ito 2001	1	-
Tarassov 2008	1	-
Gavin 2006	1 (Yih1)	0

C.1.1 Affinity purification to capture protein complexes resulted in a large number of putative YBP

The general principle of the tagged protein approach is as follows: The goal is to attach a tag to a protein, purify it using affinity purification and finally to identify the co-purifying proteins via mass spectrometry. Co-purifying proteins are in the same complex as the tagged protein, whether the interaction is direct or not. Considering that the tagged protein may be part of different complexes that consist of different proteins, this means that proteins co-purifying with a particular tagged protein are not necessarily in the same complex *in vivo* with other co-purifying proteins (Mackay et al., 2007, 2008).

Krogan et al. (2006) used tandem affinity purification (TAP) to purify protein complexes (Krogan et al., 2006). The purified proteins were then identified using both MALDI-TOF and LC-MS/MS mass spectrometry. Using two different types of mass spectrometry instead of one increases the coverage of protein-protein interactions that can be identified (Krogan et al., 2006). In addition it improves the statistical confidence of a certain interaction if it is found in both (Krogan et al., 2006). In total, 2,357 chromosomally tagged proteins were successfully purified and identified in both MALDI-TOF and LC-MS/MS and 4,087 proteins were identified with high confidence as co-complexing proteins.

The study by Krogan et al. (2006) determined a probability score for an interaction based on experimental reproducibility and mass spectrometry scores. All interactions with a probability score >0.273 were considered highest confidence interactions ("high score"). In addition the full dataset which includes non-significant interactions (<0.02, "low score") is available, in contrast to the other published studies. If proteins were of low abundance then they are more likely to be identified as non-significant. Furthermore, the current model proposes that Yih1 rapidly forms and breaks interactions depending on the cellular need, which is typical for weak transient interactions (Ozbabacan et al., 2011; Waller et al., 2012). This suggests that Yih1 could interact weakly or transiently with other YBP and that these interactions may be more likely to be lost during their experimental procedure. This would reduce the number of peptides that were available for identification in mass spectrometry and, consequently, they would be less likely to be identified as significant interaction partners. Therefore, proteins that were found in a complex with Yih1 non-significantly could still be true interactions and for this reason the low score data was included into this analysis.

In the study by Krogan et al. (2006) Yih1 was TAP-tagged. In total, 126 proteins co-precipitated with Yih1. Of those, four proteins gave a high score: Hrb1, Pci8, Tdh2 and eEF1A (the gene product of TEF1 and its paralog *TEF2*). In comparison, the number of proteins that co-precipitated with a low score is significantly higher with 122 proteins (Table C2 and Figure C1, *A*). Whether they are low or high score they were considered potential Yih1-binding proteins for this analysis (YBP). In addition, other proteins were tagged and they co-precipitated untagged Yih1. Some of the co-precipitated proteins co-resided repeatedly in the

same complex with Yih1. This raised the possibility that these additional proteins may be in the same complex with Yih1 or that they may be YBP (Figure C1, *B*). In other words, they are *indirectly* in a complex with Yih1 and thus, these are termed YBP in this work as well.

Based on the data from Krogan et al. 49 high score and 1,803 low score potential YBP were identified (Table C2). Nine tagged potential YBP co-precipitated protein complexes that contained untagged Yih1 (Figure C2). Only one of the high scored ones was found twice in a complex with tagged Yih1 and a tagged potential YBP: Tdh2 was found in a complex with Yih1 and Ilv1. In contrast, several hundreds of the low scored additional YBP were found in a complex with two or more tagged potential YBP. The maximum number was seven times, i.e. a certain additional YBP was found in a complex with seven tagged YBP and/or Yih1. Those proteins were Aap1, Pop2 and Shp1 and Figure C2 shows their distribution as co precipitators between the nine tagged YBP and Yih1. All of them were found in a complex with Sem1, Ilv1, eIF2α, Pat1 and Atp11. Of note is that these five proteins formed distinct complexes because these proteins were not co-precipitating each other for the most part.

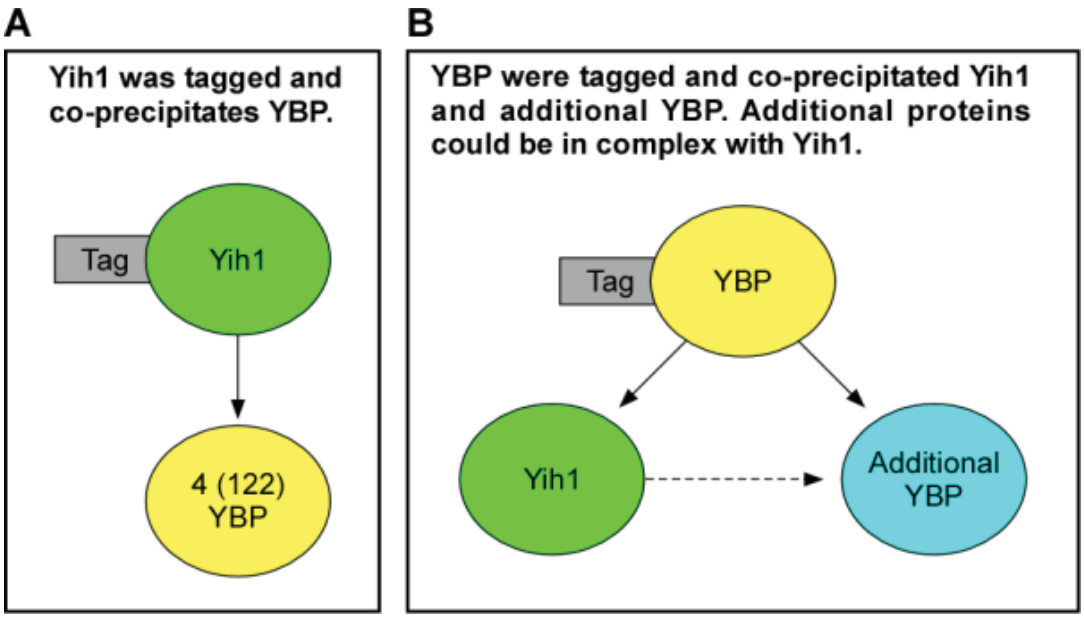


Figure C1: YBP and additional YBP are in a complex with Yih1. ***A***: Yih1 was tagged and co-precipitated potential YBP. The number of high and low scored (in parenthesis) co-precipitating proteins is given based on Krogan et al. 2006. ***B***: A tagged YBP co-precipitated Yih1 and other proteins (additional YBP) and these might be indirectly in a complex with Yih1. Therefore, they could be potential YBP.

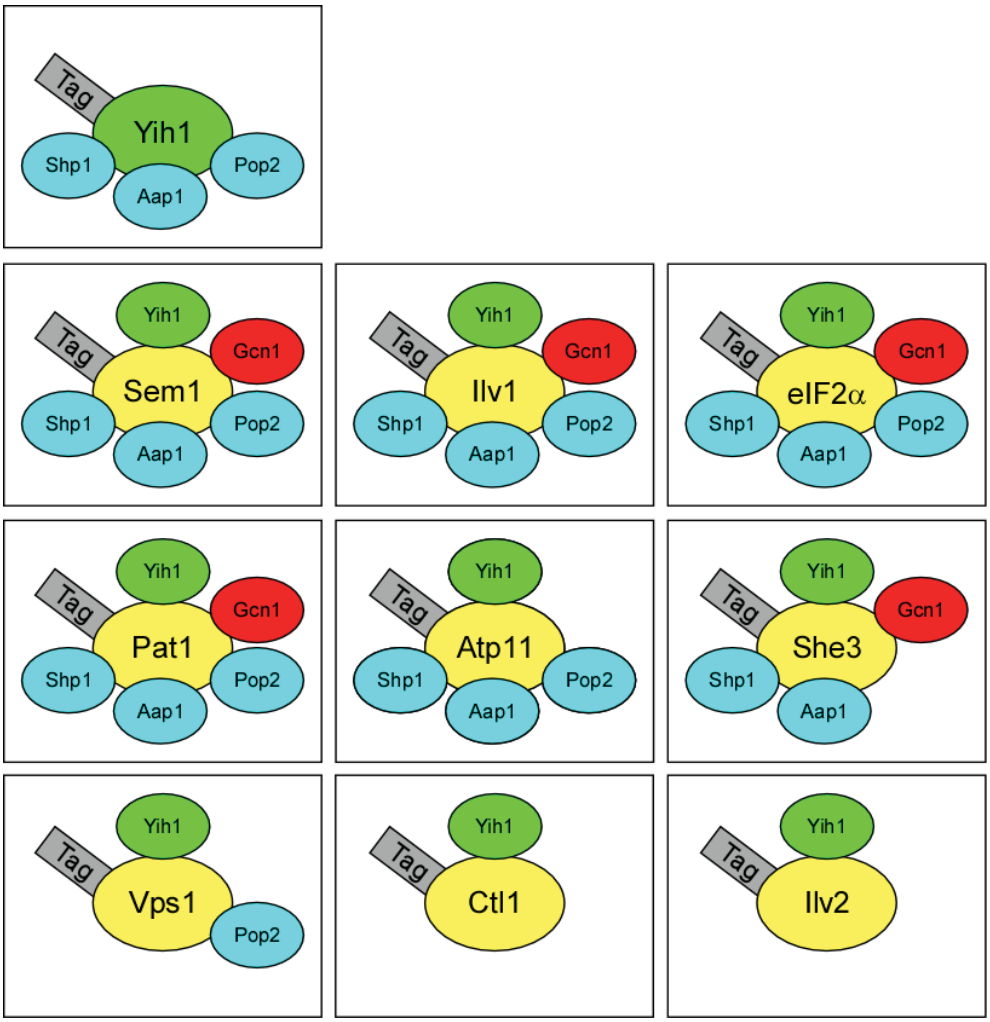


Figure C2: Example of additional YBP based on data from Krogan et al. (2006). Shown are tagged Yih1 and three YBP (*top row*) as well as the nine tagged YBP (*large ovals*) that were identified Yih1 as co-precipitators. Shp1, Aap1 and Pop2 are additional YBP (*small ovals*) and each was found in a complex with Yih1 and a combination of the nine baits. Included in this graphic is the known YBP Gcn1 which was not identified as a YBP by Krogan et al. but as an additional YBP. Note that the attachment of the "additional YBP" only indicates their presence in the same protein complex. It does not indicate a direct physical interaction because co-precipitation assays cannot be used to show this (Mackay et al., 2007, 2008).

This analysis was done for all 1,803 proteins that were identified as YBP and additional YBP (see Appendix A for the Yih1 interactome). Of the known Yih1-binding proteins, Gcn1 was not found in a complex with tagged Yih1. Instead, it was in a complex with five tagged YBP, indicating that this approach is able to identify interactions that would be missed otherwise (Figure C2). One of the tagged proteins that co-precipitated both Yih1 and Gcn1 is eIF2α (the gene product of *SUI2* which is not known to bind to Yih1), the protein that is phosphorylated by Gcn2. On the other hand, actin was not identified either as a

YBP in this study nor as an additional YBP. While a number of ribosomal proteins were identified in a complex with either Yih1 or potential YBP the previously discovered potential YBP RPS22 and RPL39 are not among them (Waller et al. 2012). These results indicate that Yih1 is found in more protein complexes than known at present and that more interactions are to be identified.

Gavin et al. 2006 also used TAP-tagging, followed by MALDI-TOF mass spectrometry. In their study 1,993 proteins were successfully tagged which is only a slightly lower number compared to the 2,357 proteins tagged by Krogan et al. (2006). However, the total number of co-purifying proteins is significantly lower at 2,760 compared to 4,087. Yih1 was TAP-tagged but no co-purifying proteins other than Yih1 itself were identified. This identification of Yih1 may be due to the identification of the tagged protein in the mass spectrometry. Additionally, Yih1 was not found to be in a complex with one of the other tagged proteins.

While the studies by Gavin et al. (2006) and by Krogan et al. (2006) applied very similar methods there are important differences. Details on the salt concentration that were used are important information since an interaction can be more or less stable depending on the salt concentration. In the method section of the Krogan et al. (2006) publication they refer to their previous work (Krogan et al., 2002) but in that study they used a range from 100 to 200 mM NaCl. Therein they report that decreasing the NaCl concentration to 100 mM resulted in a higher co-purification rate of additional proteins. Therefore, one can assume that 100 mM NaCl was used by Krogan et al. 2006. Gavin et al. used 50 mM KCl and 100 mM NaCl for their purification buffer, based on the TAP purification protocol established by Puig et al., 2001. The effect of the salt concentration on protein-protein interactions is difficult to determine and depends on the proteins involved and the type of interaction. Some proteins are not affected in their ability to form interactions by a change in salt concentration while some are (Dumetz et al., 2007). It appears that low concentration (<500 mM) of either NaCl or KCl can increase attraction between proteins in certain cases. That being said, it is not clear what influence the presence of both NaCl and KCl in one buffer has on the stability of protein-protein interactions. This is important to take into account, considering that even small changes in salt concentrations can make a significant difference in affinity purification studies, as shown by Krogan et al. (2002) (Krogan

et al., 2002). The specific effects need to be investigated for every single Yih1-YBP interaction; however, this would be difficult to achieve in considering that the interacting proteins and the type of interaction are not known yet. While it would be tempting to use the known Yih1-Gcn1 interaction as a standard this does not necessarily tell us the optimal conditions for other YBP.

Both the datasets generated by Krogan et al. and Gavin et al. are considered "high quality" (Collins et al., 2007). However, this may only be true when looking at the whole data set and not for Yih1 and YBP. Considering that Gavin et al. did not identify any known YBP it appears that the conditions they used are not optimal for finding Yih1-binding proteins (see Table C3 for a summary of the major differences). On the other hand, the study by Krogan et al. may have identified a large number of false positives, for example unspecific binding or co-complexing proteins, especially among the proteins that were low scoring. Both Krogan et al. and Gavin et al. published as supplementary data a list of proteins that they consider common contaminants in affinity purifications (Gavin et al., 2002; Krogan et al., 2004). This list includes Tdh2 and eEF1A. These proteins are considered high score Yih1 co-precipitators in the Krogan et al. (2006) study. This raises the possibility that they may truly be part of the Yih1 protein complex instead of contamination. In addition, eEF1A is already a candidate for Yih1-binding (E. Sattlegger and B. Castilho, unpublished data. See Chapter D.6 for more detail.).

The study done by Gavin et al. 2002 is similar to their 2006 study. They used the same conditions but tagged fewer proteins and subsequently found fewer co-purifying proteins. Yih1 was neither tagged nor found in a complex with other proteins.

Table C3: Major experimental differences between Krogan (2006) and Gavin (2006).

	Krogan (2006)	**Gavin (2006)**
Salt	100? mM NaCl	100 mM NaCl; 50 mM KCl
TAP incubation	3 h at 4˚C	2 h at 4˚C
Sample preparation for mass spectrometry	SDS-PAGE	NuPAGE
	Silver straining	Coomassie blue
	Visible bands cut	Whole lane cut into 1.5mm bands
Mass spectrometry	MALDI-TOF MS LC-MS/MS	MALDI-TOF MS

Babu et al. (2012) used the TAP-tagging approach as well but they concentrated on membrane-associated proteins. 1,590 proteins have been tagged and 2,875 co-precipitators were found. Yih1 is not known to associate with membranes and therefore was not tagged. Additionally, it was not found in a complex with other tagged proteins.

In the study by Peng et al. (2003) the ubiquitin protein Ubi4 was His-tagged (Peng et al., 2003). The goal was to find proteins that bind ubiquitin and to identify ubiquitination sites in proteins. Whole-cell extracts were subjected to affinity purification and then analysed via mass spectrometry. 1,075 proteins were found to co-precipitate with Ubi4 and among them is Yih1. After ubiquitination, proteins are rapidly degraded and thus can escape detection. The procedure needs to be adjusted in a way that it stabilises these interactions; however, this was not done in this study and as such the interactions found therein “represent only a subset of all ubiquitin conjugates” (Peng et al., 2003). Because the focus was on ubiquitin and ubiquitination this study is not applicable to this work. Additionally, Ubi4 was not identified as a YBP in any of the other published PPI studies. For these reasons data from this study was not included for further analysis.

Ho et al., 2002 used a FLAG-tag approach(Ho et al., 2002). They tagged 725 proteins which is only one tenth of the whole yeast proteome and which are significantly fewer than were tagged in the studies by Krogan et al. and Gavin et al. above. Yih1 was neither tagged nor identified as a co-precipitating protein.

C.1.2 Investigating interactions between two proteins resulted in few novel YBP but identified the Yih1-Gcn1 interaction

Most of the studies investigating interactions between two proteins used the yeast two-hybrid approach. This method is based on a transcription factor that consists of two domains, a DNA-binding domain (BD) and an activation domain (AD). If two proteins are fused to each domain and if those two proteins are interacting then the two transcription factor domains are brought together into close proximity. Subsequently, the transcription factor activates a reporter gene and enables the cell to grow on selection plates.

The study done by Uetz et al. (2000) used a set of about 6,000 yeast strains, each transformed with one yeast ORF fused to the activating domain (Uetz

et al., 2000). 957 interactions have been identified. *YIH1* was among the ORFs fused to the activating domain and interacted with Urh1 and Pir5.

Ito et al. (2001) tagged all yeast ORFs with either the DNA binding or activating domain (Ito et al., 2001). This resulted in 841 interaction pairs. Yih1 was fused to the activating domain and was found to interact with Jsn1.

The study by Yu et al. (2008) tested a sample of protein interactions generated by Uetz et al. and Ito et al. and experimentally evaluated the quality of their data (Yu et al., 2008). They concluded that both studies are of high quality because they performed similarly to a reference set. In addition, they performed their own large-scale yeast two-hybrid screen by investigating 1,278 proteins, resulting in 1,809 interactions. However, Yih1 was not identified.

In regards to Yih1 and YBP there is no overlap between all three yeast two-hybrid studies. Even when considering all identified protein interactions in these three studies the overlap is small: Around 1/5 of interactions found by Uetz et al. have been identified by Ito et al. Not more than 10% of the interactions identified by Yu et al. are also found by either Uetz et al. or Ito et al. This is partly a result of the low number of ORFs that were reliably expressed in each study which causes a relatively low coverage of the whole yeast proteome, indicating the yeast two-hybrid interactome for yeast is still incomplete.

Tarassov et al. (2008) conducted a protein-complementation assay (PCA) which uses a similar principle as the yeast two-hybrid: Two proteins are fused to complementary fragments of the reporter, a mutant dihydrofolate reductase (DHFR). It is insensitive against its inhibitor, methotrexate, but retains its catalytic activity (Tarassov et al., 2008). If there is an interaction between tagged proteins then the two DHFR fragments come together and, in turn, the DHFR activity is reconstituted, allowing the cells to grow on methotrexate-containing plates. In this study, 5,367 ORFs were successfully tagged and 2,770 protein interactions were established. Yih1 was tagged and Gcn1 was identified as the only interaction partner. This is the only study that identified one of the two known YBP.

C.1.3 Discussion

The study done by Krogan et al. (2006) identified the majority of the potential YBP of all published large-scale interactome studies (cf. Table C2). This is partly because they provided low scoring interactions as supplementary data.

Despite this, they did not identify the known Yih1-binding protein actin while Gcn1 was only found indirectly in a complex with other YBP. One explanation might that the TAP tagging method that they used is more likely to miss weak or transient interactions because of the two-step procedure which leads to a high dilution of the protein solutions in the later steps (Gavin et al., 2002; Tagwerker et al., 2006; Xu et al., 2012).

Some proteins co-precipitated more than once with Yih1, either directly with TAP-Yih1 or indirectly with one of the tagged proteins that co-precipitated Yih1 (cf. Figure C2). These are strong candidates for novel YBP. As they are found relatively frequently in a complex with Yih1 compared to other proteins they are more likely be YBP or proteins that are involved in Gcn2 regulation.

Both Krogan et al. (2006) and Tarassov et al. (2008) found the known Yih1-binding protein Gcn1 and, of all proteins that were found in a complex with Yih1, this is the only protein that was identified in more than one study. Curiously, the proteins Jsn1, Pir5 and Urh1 were only found as Yih1 interactors by the two yeast two-hybrid studies (Uetz 2000, Ito 2001). They were not found in the affinity purification studies, not even as low scoring proteins. These may suggest that both yeast two-hybrid approaches identified different subsets of protein-protein interactions or a high number of false positives. On the other hand, the affinity studies may show a large coverage but at the same time a low sensitivity and thus might have missed these interactors. These findings are an indication that not all Yih1-binding proteins have been found so far.

One known caveat with affinity assays is that they cannot be used to determine if there is a direct physical interaction between proteins (Mackay et al., 2007, 2008). A co-precipitating protein may not directly interact with Yih1 but instead it may be connected via one or more bridge proteins. In addition, there is the possibility of false positives due to the formation of protein complexes that may not exist in a native environment or due the inclusion of promiscuous proteins. Other methods such as the yeast two-hybrid system may be more informative in regards to determining direct interactions (Yu et al., 2008). However, a YBP that does not directly interact with Yih1 may still be involved in promoting Gcn2 function, for example via a mediating protein (Figure A8).

Proteins that are not known to bind Yih1 but are involved in the GAAC were identified. Only Gcn3 (eIF2Bα) (Hannig and Hinnebusch, 1988) and eEF1A (Visweswaraiah et al., 2011b) were found in a complex with a tagged Yih1 while Gcn1, Gcn2, Gcn20, eEF3 and eIF2α were found only indirectly with Yih1 by co-precipitating with other tagged YBP. Gcn2 and Gcn20 might be expected to co-precipitate because they are binding Gcn1 and indeed, they were only found in a complex that also contained Gcn1. Similarly, eIF2α is binding Gcn2 and it was co-precipitating Gcn2 and Gcn1. Gcn20 and eIF2α were only found once in a complex with Yih1, suggesting a weak interaction with its respective binding partners. Other proteins that are involved in the GAAC were identified more than once with Yih1, such as eEF1A and Gcn3 (eIF2Bα). eEF1A is highly abundant and is often considered a contaminant in affinity purification studies. However, there is already evidence that it may be in a complex with Yih1 (E. Sattlegger and B. Castilho, unpublished data). In addition, as it is binding Gcn2 and is involved in the GAAC it was considered a putative YBP (Visweswaraiah et al., 2011b). Additionally, eEF3 was found in a complex with tagged Ilv1 and Yih1. This suggested that Yih1 could perform its Gcn2-inhibitory function not only by binding Gcn1 but also by binding other proteins involved in protein translation. For example, it was proposed that eEF1A may deliver uncharged tRNA to Gcn2 or that this is mediated by Gcn1 (Visweswaraiah et al., 2011b). Thus, Yih1 may inhibit this process by binding to eEF1A, Yih1 may recruit another factor, or if Gcn1 is involved then Yih1 could inhibit the ability of Gcn1 to transfer tRNA to Gcn2 (Sattlegger et al., 2004).

Three proteins were most frequently found in different complexes that contained Yih1 (Figure C2). Aap1 acts as a positive regulator of glycogen accumulation (Caprioglio et al., 1993). Under histidine starvation conditions its expression is induced by Gcn4 (Natarajan et al., 2001), indicating that it has a role in the GAAC. However, as this role is downstream of Gcn4 it is likely not important for Gcn2 function, although the possibility that it may be a YBP but without regulating Gcn2 cannot be excluded. Pop2 is involved in mRNA degradation (Daugeron et al., 2001). Degradation of mRNA and translation are in a balance under replete conditions. In the case of amino acid starvation the level of mRNA for proteins involved in the starvation response is increased (Gasch et al., 2000). It

is conceivable that when a cell senses the starvation the activity of Pop2 (and other factors) is increased in order to recycle mRNA for the synthesis of mRNA for starvation response proteins. As Yih1 is repressing the starvation response it may act as a Pop2 inhibitor under replete conditions by binding to it and is released under starvation conditions. As strains without Yih1 do not show a growth defect this may involve other factors or Yih1 is only involved under certain conditions or to a lower extent in this pathway. Shp1 is involved in mitosis and is associated with the proteasome due to its ability to bind ubiquitylated proteins. Interestingly, Shp1 positively regulates Glc7, a protein phosphatase that acts in opposition to Gcn2 in that it reduces eIF2α phosphorylation (Wek et al., 1992). Thus, a Yih1-mediated activation of Shp1 may allow a reduction of the GAAC by reducing the level of eIF2α-P.

In summary, half of the large-scale protein-protein interactions studies have not identified Yih1 either as a co-precipitating protein in affinity studies or as an interactor in yeast two-hybrid and protein complementation assays. Between all five studies that identified YBP there was no overlap in identified putative Yih1-binding proteins. In addition, the known YBP actin and Gcn1 were not found or were in a complex with Yih1 only indirectly, respectively. This underscored the incompleteness of the Yih1 interactome.

C.2 Conduct experiments in-house to specifically find YBP

The goal is to establish an experimental procedure for identifying proteins that are in complex with Yih1.

C.2.1 Formaldehyde crosslinking as a potential tool to stabilise protein interactions

The chemical formaldehyde can stabilise protein-protein interactions crosslinking amino acid residues from both binding partners. Formaldehyde is a small molecule (Figure C3) that quickly penetrates the yeast cell wall and crosslinks proteins. Formaldehyde has a number of features that make it useful for this work (Klockenbusch and Kast, 2010): It is a small molecule and therefore it crosslinks only proteins that are in close proximity (up to 2.7 Å), in other words those proteins that are likely to interact. Since it is fast-acting it can take a snapshot of the native conditions inside a cell at a certain point in time. Regulatory proteins such as the ones that are involved in the starvation response often show weak or transient interactions because they are finely tuned by necessity for their regulatory function. Formaldehyde would crosslink these interacting proteins and allow them to withstand the purification procedure (Tagwerker et al., 2006). In addition, as crosslinking retains the native cellular conditions it would reduce the number of unspecific interactions (i.e. false positives) that result from the mixing of proteins from different cell compartments during cell lysis (Mackay et al., 2008). Another advantage of using formaldehyde is that the crosslinking can be reversed which is important for downstream applications (such as Western blotting). Finally, while it modifies a protein by attaching to it this has no known effect on protein identification by mass spectrometry due to its small molecular size and formaldehyde can be easily excluded from the data. In order to crosslink proteins with formaldehyde the side chains of lysine and tryptophan need to be available (Toews et al., 2008). Therefore, the amino acid contents influence the crosslinking success and subsequently influence the extent of the stabilisation of protein-protein interactions.

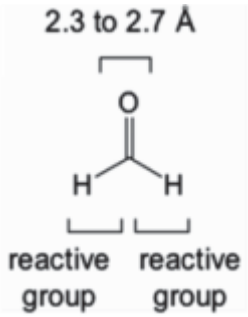


Figure C3: Chemical structure of formaldehyde (taken from Sutherland 2008).

C.2.2 Formaldehyde crosslinking using Yih1 at native levels

A myc-tagged Yih1 expressed from a low copy plasmid was used to test the suitability of crosslinking Yih1 at native levels. While there are a variety of formaldehyde conditions to be found in the published literature, concentrations of more than 1% and incubations times longer than 1 h are agreed upon to not improve protein-protein crosslinking efficiency or even have a negative effect on protein concentration in the whole cell extract (e.g. (Jackson, 1999; Klockenbusch and Kast, 2010; Sutherland et al., 2008; Vasilescu et al., 2004)). At the same time it seems that 0.3% to 0.5% at room temperature (RT) can lead to a good balance between protein loss and recovery (Sutherland et al., 2008). Reasons for the protein loss include loss of protein due to insolubility, precipitation at higher concentration and incubation times or formation of crosslinks with DNA. Based on this the formaldehyde concentrations was kept inside the same range in these optimisation studies.

Yeast cells expressing myc-Yih1 (pES333-2-9, Table B4) were grown to exponential phase in 300 mL culture of appropriate media at 30˚C. This was followed by crosslinking using 1.0% formaldehyde and no crosslinking at room temperature (RT) or at 4°C for 10 min or 60 min. Cells were subsequently lysed and equal amounts of whole cell extract were separated by SDS-PAGE and analysed in a western blot (Chapter B) using antibodies against the myc tag and actin. As shown in Figure C4 myc-Yih1 was present in all non-crosslinked samples. However, in the crosslinked samples Yih1 was only detected when the crosslinking was done for 60 min at 4°C (*lane 7*) and for 10 min at RT (*lane 1*, longer exposure). In other words, the crosslinking procedures resulted in a fewer proteins that were available for antibody binding. This suggested that the

formaldehyde negatively affected protein concentration, possibly due to a precipitation of myc-Yih1 inside a larger protein complex during the sample preparation. A myc-Yih1 signal is present in samples that were crosslinked at 4˚C for 1h but not when crosslinked for 10 min. The presence of actin was tested and, in contrast to Yih1, it was equally present under all conditions except in samples that were crosslinked at RT for 60 min (*lane 3*). The results shown in Figure C4 indicated that, at room temperature, 10 min crosslinking could be sufficient. However, while the actin concentration in the whole cell extract was not affected by the formaldehyde the Yih1 protein concentration was reduced. It was reasoned that this was due to the high formaldehyde concentration used which is known to reduce protein levels (Jackson, 1999; Klockenbusch and Kast, 2010; Sutherland et al., 2008; Vasilescu et al., 2004).

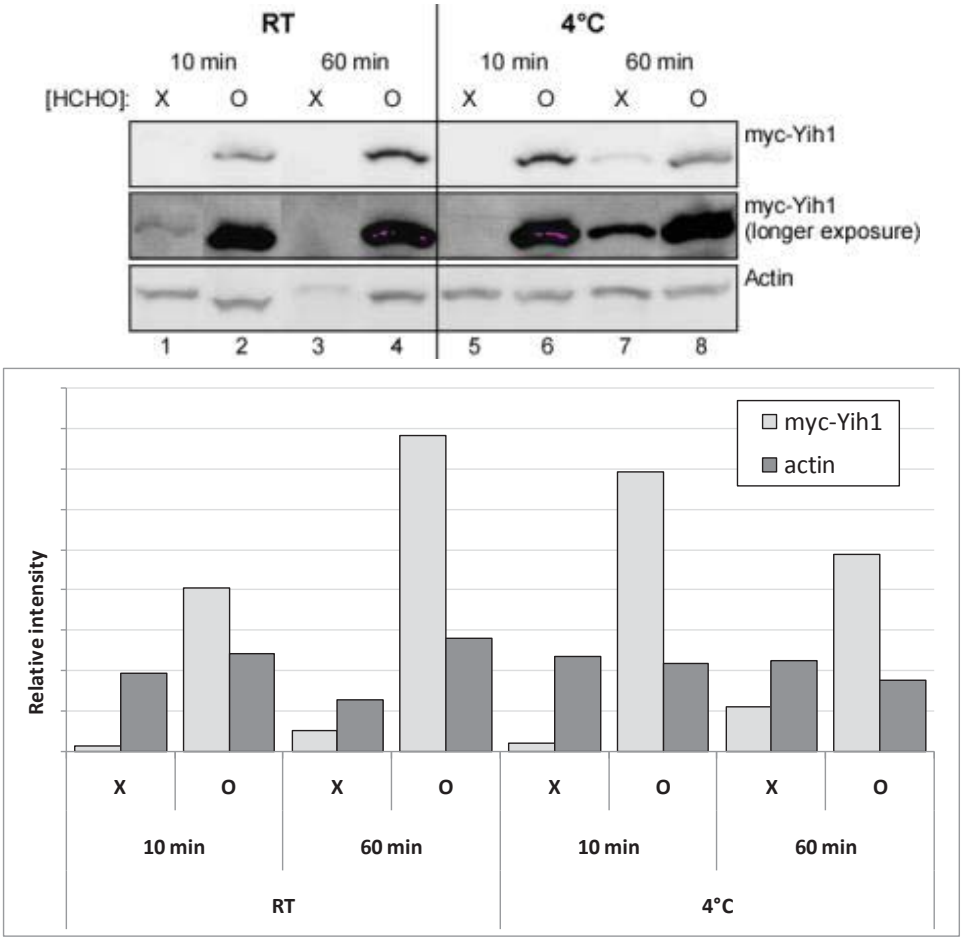


Figure C4: Myc-Yih1 is detected when the crosslinking was done at RT for 10 min and at 4°C for 1h. Cells grown to exponential phase were treated with 1% formaldehyde at different crosslinking durations and temperatures as indicated. Yeast whole cell extract containing a myc-tagged Yih1 was resolved with SDS-PAGE and subjected to immunoblotting using antibodies against myc and actin. HCHO: 1% formaldehyde (v/v). X and O: crosslinked and non-crosslinked, respectively. RT: room temperature.

C.2.3 myc-Yih1 at native levels does not co-purify Gcn1 or actin

The next question was whether the formaldehyde negatively affects the affinity purification of myc-Yih1 and the co-purification of proteins. It is known that Yih1 directly binds Gcn1 as well as actin (Sattlegger et al., 2004). This interaction is used as an indicator for whether these interactions were preserved by formaldehyde crosslinking. In addition, if Gcn1 and actin are co-precipitating with Yih1 under non-crosslinking conditions then this would indicate a successful pulldown. The treatment of cells with formaldehyde prior to the pulldown can tell us whether it stabilises this interaction or reduces the amount of protein that is pulled down. Of note is that Gcn1 has not yet been observed to co-precipitate with Yih1 at native levels and therefore it is possible that it might not be seen it in these experiments.

For these studies, cells expressing myc-Yih1 and vector-only control were grown to exponential phase, followed by formaldehyde crosslinking. For the affinity purification 0.5 mg of whole cell extract protein were incubated with anti-myc agarose beads for 2 h (Chapter B.12). After washing the beads (which bound myc-Yih1) they were boiled in presence of loading buffer and the supernatant was separated via SDS-PAGE. Western blot was used to visualise myc-Yih1, Gcn1 and actin.

The myc signal intensities in the input control for 0 to 0.5% of formaldehyde were of similar strength (Figure C5, *lanes 8-11).* Only for 1.0 % there was a marked decrease (*lane 12*).

As expected, no myc signal was seen for the vector control (*lane 7*). Myc-Yih1 co-precipitated under non-crosslinking conditions and with 0.1% formaldehyde (*lanes 2 and 3*). There was no signal for 0.3 to 1.0% formaldehyde (*lanes 4-6*). This might be because the formaldehyde reduced antibody binding to myc-Yih1. However, this was not the case for the respective samples in the input control (compare the signal ratios in *lanes 3 and 9* to the signal ratios of *lanes 4 and 10*, for example). Another explanation was a faulty protein transfer from the SDS-PAGE gel onto the membrane. A small part of the myc-Yih1 signal for 0.1% samples was missing (*lane 3*), a typical sign that in his region of the membrane the protein was not fully transferred.

Gcn1 was detected only for the vector-containing input and precipitate (*lanes 1 and 7*), indicating unspecific binding of Gcn1 to the beads. No Gcn1 signal was detected for the input and the immunoprecipitate containing myc-Yih1 (*lanes 2-6 and 8-12*). This was unexpected because Gcn1 should be present at least in the input control. It is possible that the expression level of Gcn1 in these strains was too low to be detected. Supporting this idea was that the overall signal intensity was relatively low. It is known that the location of a protein tag can negatively affect protein folding and function and that this could be another explanation why Gcn1 is not binding (Halliwell et al., 2001; Terpe, 2003). However, this was unlikely because the myc-Yih1 has been shown previously to not affect Yih1 function (although the possibility cannot be excluded that the tag has other unknown negative effects that can influence protein-protein interactions) (Waller et al., 2012). Therefore, it cannot be confirmed if Gcn1 co-precipitated with Yih1 in this assay.

For actin, there was a signal in the input control (*lanes 7-12*) and there was a decrease in signal intensity for the samples incubated with 1.0% formaldehyde (*lane 1-2*), similar to the myc signal. No actin co-precipitated with myc-Yih1 (*lanes 2-6*). There was a weak actin signal for 1.0% formaldehyde (*lane 6*); however, this was likely due to a sample spill-over from the neighbouring gel pocket (*lane 7*) during the SDS-PAGE loading.

In conclusion, the interaction of Yih1 with Gcn1 and actin when Yih1 was expressed at native levels was not verified. In addition, it was not possible to determine if formaldehyde has stabilised these interactions.

C.2.4 Using Yih1 at overexpressed level for formaldehyde crosslinking

It was tested if formaldehyde crosslinking is able to stabilise the Yih1-Gcn1 interaction. As the Yih1 concentration was insufficient when it was expressed at native levels, Yih1 at overexpressed levels was used instead in order to increase the number of interactions.

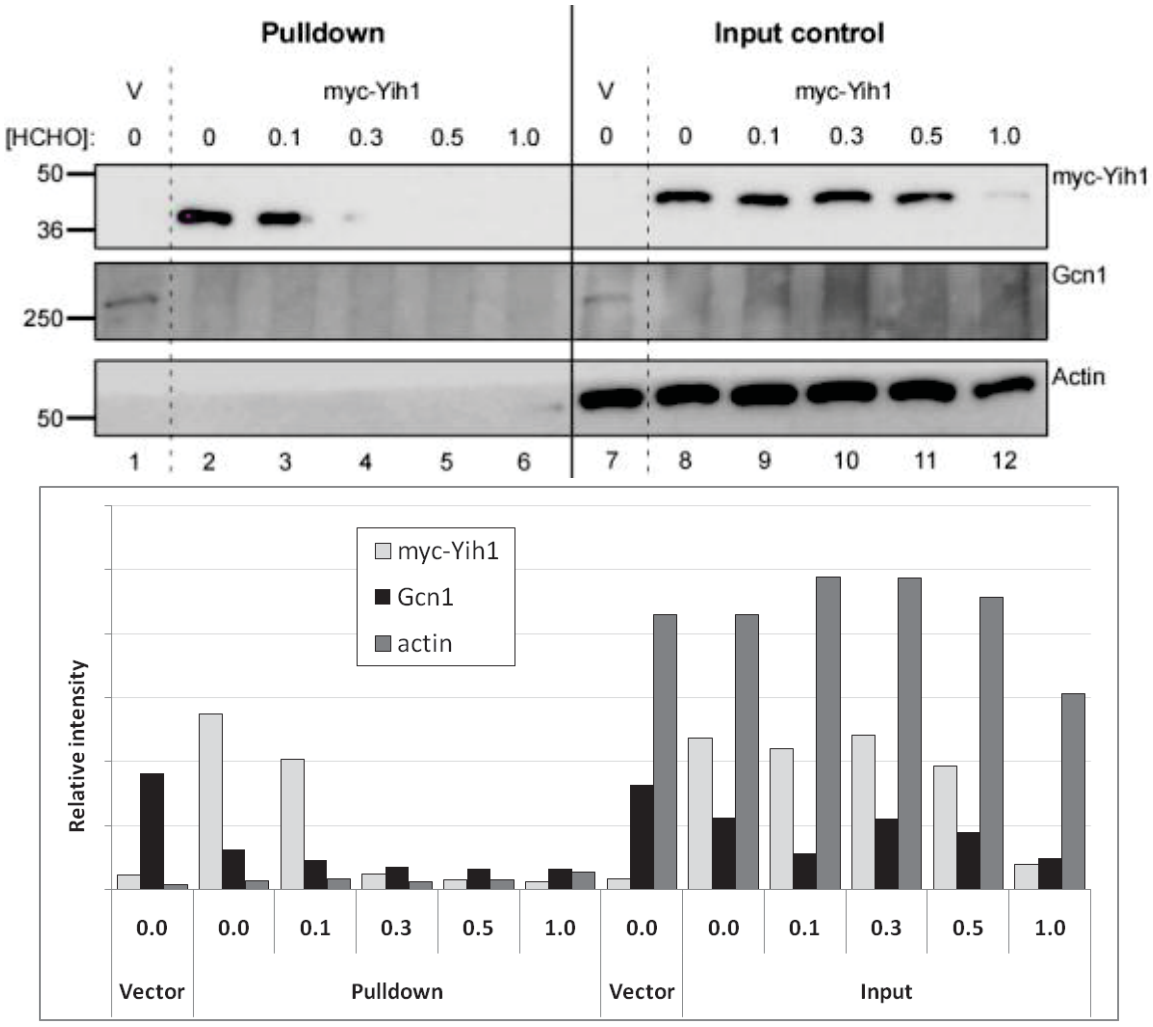


Figure C5: Yih1 at native levels does not co-precipitate Gcn1 or actin in a myc affinity purification. Formaldehyde concentrations used are as indicated. V: vector control.

In vitro crosslinking of GST-Yih1 at overexpression levels resulted in the formation of slowly migrating protein complexes

In this experiment a GST-tagged Gcn1 fragment (GST-Gcn1[2052-2428]) was used that is known to bind Yih1 and a His_6-tagged Yih1 (Sattlegger et al., 2011). Both were separately overexpressed in *E. coli*. A GST pulldown using glutathione beads was performed to purify the Gcn1 fragment from the bacterial extract. This was followed by incubating these beads with His_6-Yih1. 1.0% of formaldehyde at room temperature was used in order to maximise the likelihood of crosslinking. If so, then crosslinking should result in the formation of additional, slower migrating bands at a higher molecular weight in a western blot (Figure C6). Boiling these samples at 95˚C reverses the crosslinks and the additional bands

should disappear, confirming that the bands of larger molecular weight are crosslinked protein complexes (Klockenbusch and Kast, 2010; Vasilescu et al., 2004). Additionally, a low temperature at which crosslinks remained intact was used. Protein-DNA crosslinks are commonly reversed at 65˚C (Boyd and Farnham, 1999; Jackson, 1988; Kuras and Struhl, 1999; Orlando et al., 1997). Therefore, in order to ensure that the crosslinks are not destroyed samples were treated at 55˚C, in parallel to 95˚C.

The molecular weight of the Gcn1 fragment appears to be 70 to 80 kDa, based on previous studies (Sattlegger and Hinnebusch, 2000). The size of His_6-Yih1 is around 45 kDa (Sattlegger et al., 2011). An interaction between the Gcn1 fragment and Yih1 would therefore result in a complex of around 120 kDa. The fact that the crosslinks were not fully destroyed at 55˚C and that this may change the migration pattern to some extent, depending on the proteins that are present in a crosslinked protein complex, needs to be taken into account (Fowler et al., 2011).

A relatively strong signal of around 70 to 80 kDa that was visible on a Coomassie blue stained gel and an anti-GST western blot corresponds to the GST-Gcn1 fragment, under both crosslinking and non-crosslinking conditions as well as at 55˚C and 95˚C (Figure C7 top and bottom, *lanes 1 to 4*). This indicated that the GST-Gcn1 fragment was pulled down successfully. GST alone was also pulled down successfully under all conditions (top and bottom, *lanes 5 to 8*).

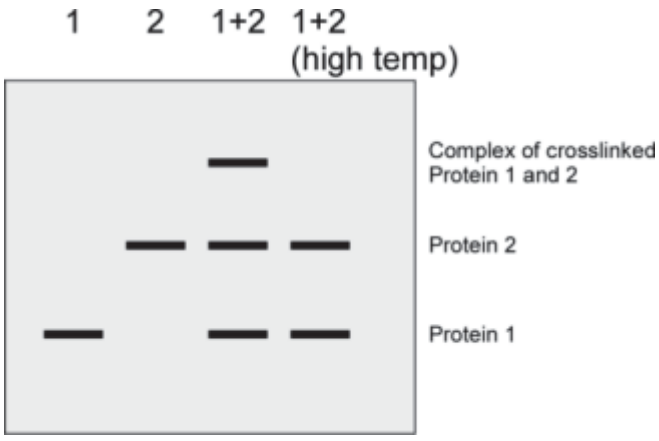


Figure C6: Schematic of a western blot showing the formation of additional bands that consist of a stable complex of protein 1 and 2 when incubating them with formaldehyde as well as the crosslink reversal at high temperature.

The signal corresponding to His_6-Yih1 is detected at around 45 kDa in Gcn1 fragment-containing samples on the Coomassie stained gel and Western blot (*lanes 1-4*). This indicates that it was specifically co-precipitated under all conditions with GST-Gcn1[2052-2428] but not with GST alone (middle, *lanes 1-4*). This indicates that Yih1 binds Gcn1, as was published before (Sattlegger et al., 2004, 2011). It was possible that the signal seen was simply an indicator for a GST degradation signal, seen as several bands below 64 kDa in the anti-GST western blot (top, *lanes 1-4*). However, this is unlikely because 1) the molecular weight of Yih1 signal on the Coomassie-stained gel corresponds to the signal on the anti-His western blot and 2) the band between 36 and 50 kDa is only seen on the Coomassie stain but not on the anti-GST western blot.

As seen on the Coomassie stained gel the addition of formaldehyde resulted in the formation of at least one visible additional band at a molecular weight above 148 kDa (Figure C7 bottom, *lane 1*). The GST antibody detected this band and others below 148 kDa in the western blot, suggesting that these bands contain the GST-Gcn1 fragment (top, *lane 1*). Western blotting can be more sensitive than Coomassie staining and may explain why more bands were detected with this method. These bands were not seen in the non-crosslinked samples (top and bottom, *lane 2*). This suggested the presence of a crosslinked complex that is migrating slower than the GST-Gcn1 fragment. In addition, boiling the samples at 95˚C resulted in a reduction in signal intensity of the bands seen with Coomassie staining and the anti-GST western blot (top and bottom, *lane 3*). This suggested that the additional bands contained protein complexes that were stabilised by formaldehyde crosslinking.

His_6-Yih1 was detected in a western blot at the expected molecular weight (Figure C7 middle, *lanes 1-4*). It was expected that the additional bands are seen at around 120 kDa which would correspond to a crosslinked and stabilised Yih1-Gcn1 fragment complex. However, no additional bands of any size were detected. This could indicate that the amount of His-Yih1-Gcn1 fragment complexes was too low to be detected by the His antibody in this western blot. This could also be an indication that the interaction between Yih1 and the Gcn1-fragment was not stabilised by formaldehyde crosslinking. There was a His-signal at the top of the membrane. However, this was unlikely a crosslinked protein

complex because it was seen under crosslinking and non-crosslinking conditions. Additionally, in the anti-GST western blot this signal was only detected for one sample that contained the Gcn1-fragment and that was crosslinked and heated to 55˚C (*lane 1*).

As seen in the anti-GST western blot and to some extent on the Coomassie stained gel (Figure C7 top and bottom, *lane 5*) crosslinking GST alone resulted in several additional bands. These disappeared after boiling of the samples and indicated the stabilisation of crosslinked GST-containing protein complexes (top and bottom, *lane 7*). Since the level of His-Yih1 is very low (middle, *lanes 5-8*) it is unlikely that these additional bands are a result of a GST–Yih1 interaction. It is known that GST forms dimers (Fabrini et al., 2009; Kaplan et al., 1997). This would affect the interpretation of the additional bands for the Gcn1 fragment: Since Gcn1 is GST-tagged any additional bands of larger molecular weight that are seen on the anti-GST western blot or the Coomassie staining might be caused by GST multimers.

Adding formaldehyde resulted in a small reduction of signal intensity when comparing crosslinked and non-crosslinked samples (e.g. the GST signal in *lane 1* vs. *lane 2*). The cause for this could be a loss of protein due to protein aggregate formation that gets lost during the cell lysis and purification procedure. Or a certain amount of GST-Gcn1 protein formed large crosslinked protein complexes, reducing the amount of non-crosslinked protein that was detected.

To conclude, GST-Gcn1[2052-2428] co-precipitated His_6-Yih1, as published previously. The results shown in this work suggested that formaldehyde can stabilise protein-protein interactions in general *in vitro*, as evidenced by the presence of large crosslinked protein complexes. However, this procedure did not generate a crosslinked Yih1-Gcn1 fragment complex because Yih1 was not detected in these complexes.

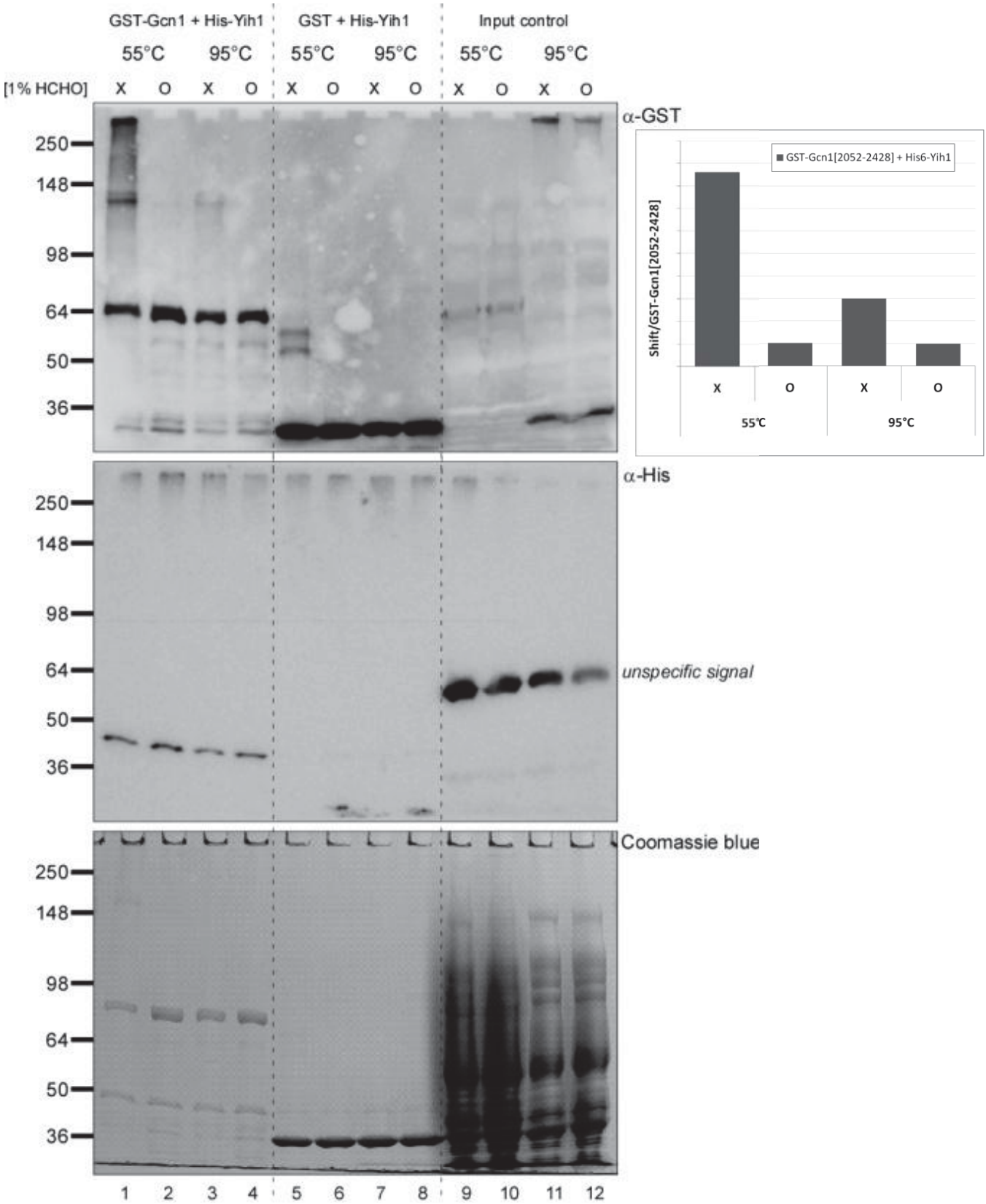


Figure C7: GST-Gcn1[2052-2428] co-precipitated His_6-Yih1 and the presence of high molecular weight bands indicates that crosslinking stabilised protein complexes. Western blots using antibodies against GST (*top*) and Coomassie staining (*bottom*) revealed the presence of additional bands in lane 1 which disappeared at 95 °C in lane 3. No additional bands were seen in the non-crosslinked samples and in the anti-His western blot (*middle*).

In vivo crosslinking of GST-Yih1 at overexpression levels resulted in the formation of slowly migrating protein complexes

Since co-precipitation of Gcn1 and actin with Yih1 expressed at native levels *in vivo* was unsuccessful, overexpressed Yih1 was used in order to drive interactions. The GST tag for Yih1 was used for these assays as it has been used successfully before for Yih1 in the Sattlegger group (see Chapter A). The GST Yih1 was expressed from a plasmid containing a galactose-inducible promoter. GST tagged proteins can easily be purified using glutathione agarose beads and then subjected to western blotting using a commercially available GST antibody that has a high affinity to GST.

Again, it was tested if formaldehyde crosslinking of whole cell extracts stabilises the Yih1-containing complexes and if this resulted in the formation of additional bands at a higher molecular weight in a western blot. In addition to 95˚C and 55˚C, 65˚C was included in this set of experiments to further investigate the stability of the crosslinked protein complexes. The formaldehyde concentrations used were 0.3 %, 0.5% and 1.0%. Ponceau S staining was used to visualise the overall migration pattern of the whole cell extract (representative result in Figure C8, *upper part*). There was no obvious difference in protein content and the number of additional bands at a higher molecular weight when comparing 55˚C and 65˚C. It was evident that the protein concentration in the 1.0% formaldehyde samples was reduced compared to 0.5% (*lane 4* vs. *lane 3*, for example). This was likely due to a loss of proteins during the cell lysis procedure due to the formation of large crosslinked precipitates. In addition, the overall protein concentration for the 95°C samples (*lanes 9-12*) appeared to be lower than for 55°C and 65˚C. This was probably due to unequal amounts of protein that were loaded onto the SDS-PAGE.

Probing for GST revealed a strong band at above 64 kDa, corresponding to the size of GST-Yih1, as well as several lower bands that were probably degradation products (Figure C8, bottom). No obvious difference in GST signal intensity was detected between 55˚C and 65˚C.

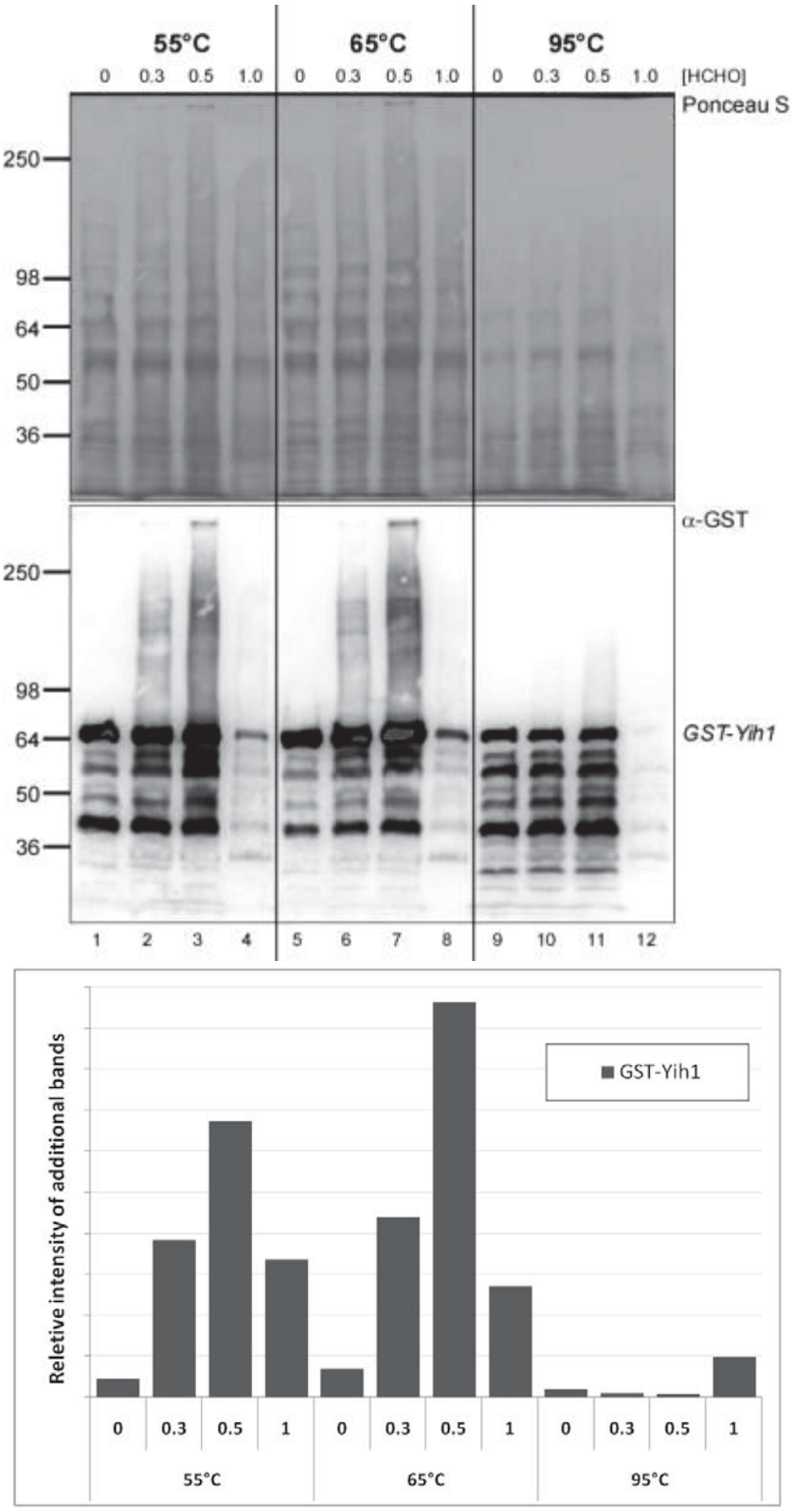


Figure C8: Formaldehyde crosslinking of yeast whole cell extract containing overexpressed GST-Yih1 results in the formation of additional bands at higher molecular weight which partly disappear after boiling the samples at 95°C. Shown are the staining with Ponceau S (*top*) and an anti-GST western blot (*bottom*). For the bottom graph the signal intensities of the two additional bands and GST-Yih1 were first normalised to the signal of the whole lane and the ratio of additional bands to GST-Yih1 was calculated.

Similar to the Ponceau S staining the signal intensity for GST-Yih1 was reduced for samples treated with 1% formaldehyde, supporting the idea that exposure to high concentrations of formaldehyde results in protein loss (bottom, *lane 3* vs. *lane 4* or *lane 7* vs. *lane 8*). The high protein concentration for 0.5% was also clearly visible (bottom, *lanes 3 and 7*).

Additional bands at a higher molecular weight were detected for 0.3% and 0.5% formaldehyde for both 55˚C and 65˚C in the anti-GST western blot (bottom, *lanes 2 and 3* and *lanes 6 and 7,* respectively), indicating that the crosslinks remained sufficiently stable at up to 65˚C. Treatment with 0.5% HCHO resulted in the highest concentration of additional bands (*lanes 3 and 7*). These bands were not present at 95˚C (*lanes 10 and 11*), indicating that the additional bands were stabilised crosslinked protein complexes. No additional bands were detected for the samples that were treated with 1% formaldehyde (*lanes 4, 8 and 12*). This was partly due to overall lower signal intensity for the samples that were boiled at 95˚C and partly due to the high formaldehyde concentration that reduces the protein concentration.

The possibility that the additional bands at a high molecular weight were due to GST multimerisation cannot be excluded. Therefore, in another experiment samples containing GST-Yih1 and GST alone were compared in regards to the presence of additional bands (Figure C9). The band patterns for GST-Yih1 appeared similar to the previous result: Additional bands were only seen under crosslinking conditions while at the same time the signal intensity for GST-Yih1 was lowest at the highest formaldehyde concentration (1.0%) (*lanes 9-12*). The molecular range of these bands was similar to the ones seen in the previous experiment. Contrary to the previous experiment, additional bands were detected for samples treated with 1% formaldehyde (*lane 12*). Relative to GST-Yih1, the signal intensity of the two additional bands below 250 kDa positively correlated with increases in formaldehyde concentration (*bottom graph*). Boiling the samples partly reduced the signal intensity for the additional bands by 40-80%. This suggested that GST-Yih1 containing protein complexes were stabilised through crosslinking and that the heat reverted the crosslinks. Unexpectedly, boiling of non-crosslinked samples increased the relative signal for the additional bands,

even though the heat was expected to reduce the signal intensities for the additional bands the strongest of all samples. The possibility of unequal loading can be excluded because the signals for GST-Yih1 and the additional bands were normalised to the whole lane. One possibility was that at least for the non-crosslinked samples the two additional bands are indicative of a stabilised GST-Yih1 protein complex. As the GST antibody was polyclonal it may have bound to a suitable epitope on different proteins instead. However, this does not explain why their signal increased or the reduction in signal intensity for the crosslinked samples after boiling.

In this experiment a band was detected at the top of the membrane for GST-Yih1-containing samples and its intensity increased together with the increase in formaldehyde concentration (Figure C9). These bands disappear almost completely with boiling and this indicated the reversal of crosslinking. In addition, this suggested that formaldehyde crosslinking resulted in the formation of a large and slowly migrating protein complex, possibly together with DNA. It was possible that, due to their large size, some of these complexes were not fully destroyed at high heat. Therefore they may have appeared together with the additional bands a below 250 kDa and subsequently may have masked the signal decrease. Therefore, longer boiling may be needed to fully reverse the crosslinking.

GST alone is present at the expected molecular weight at below 36 kDa (*lanes 1-8*). An increase in formaldehyde concentration resulted in an increase in the signal intensity of the additional bands and smear for GST samples (*lanes 1-4*). Thus, for the GST-Yih1 containing samples the high molecular weight bands may be due to the GST tag forming Yih1 multimers.

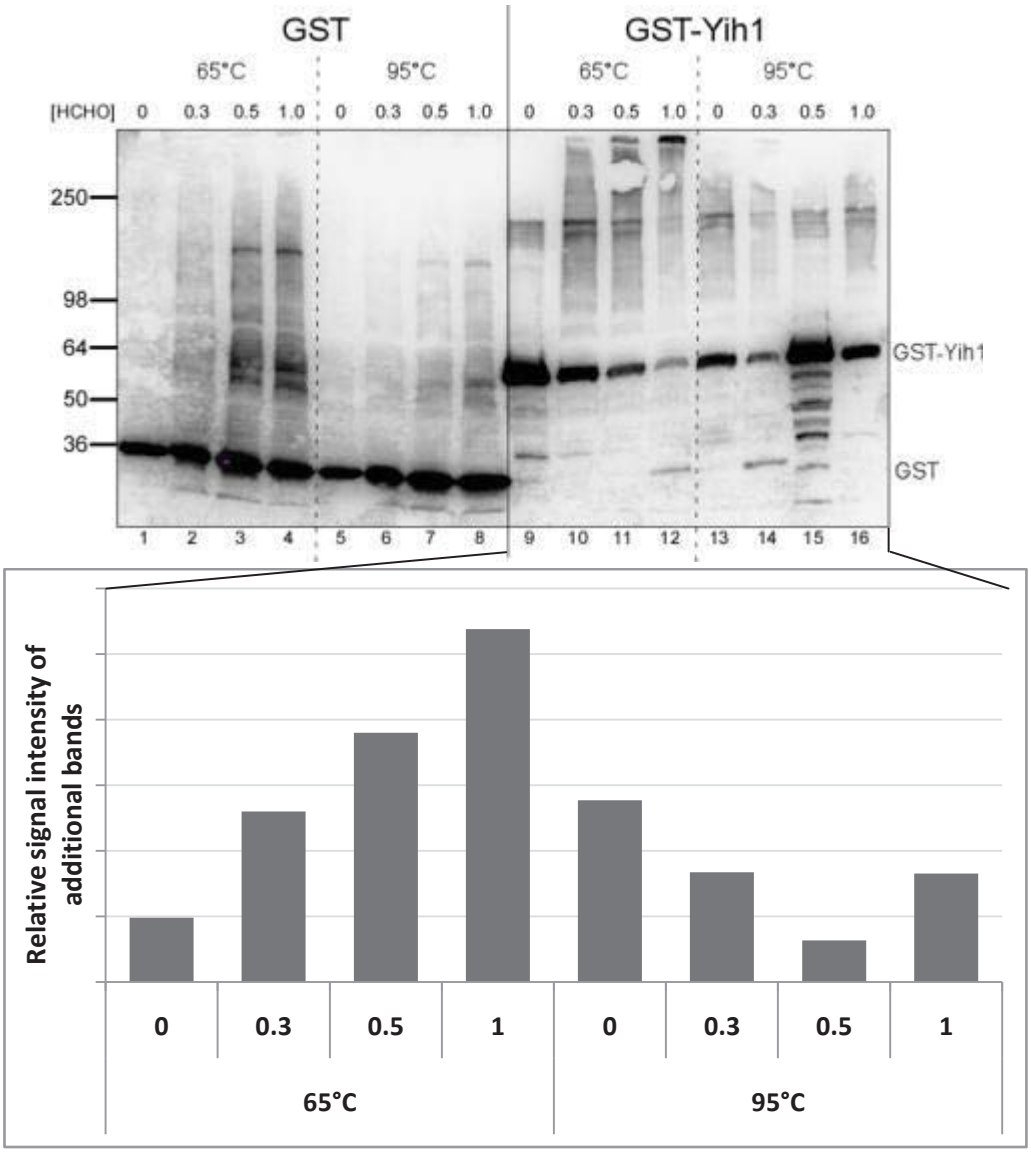


Figure C9: Formaldehyde crosslinking of yeast whole cell extract containing overexpressed GST-Yih1 results in the formation of additional bands at higher molecular weight which partly disappear after boiling of the samples at 95°C. For the bottom graph the signal intensities of the two additional bands (shift) or GST-Yih1 were first normalised to the signal of the whole lane and the ratio of additional bands to GST-Yih1 was calculated.

C.2.5 GST-Yih1 at overexpressed level co-precipitated Gcn1

Next it was investigated whether formaldehyde crosslinking affects the GST mediated affinity purification of GST-Yih1. For this, the same conditions as before were used, that is up to 1.0% formaldehyde as well as comparing sample boiling and non-boiling. A representative result is shown in Figure C10 (note: input is not shown).

Both GST and GST-Yih1 were successfully precipitated (*top*). The signal for GST was partly cut off at the bottom because it did not transfer onto the PVDF membrane but it was consistently and repeatedly precipitated in other experiments. GST was also seen on a Coomassie stained gel using part of the same samples (*bottom*). The protein concentration in the whole cell extract in the previous experiments decreased with increase in formaldehyde concentration. Therefore, an attempt was made to equalise the protein concentration by loading a larger volume of whole cell extract. However, equal loading was not achieved and the protein concentration remains lowest for 1% formaldehyde (top, *lanes 5-8*). Additional bands at higher molecular weight were present under crosslinking conditions but their signal intensity was low. Therefore, the upper part of the membrane needed a longer exposure to improve the signal quality. In agreement with previous results sample boiling at 95°C lead to a decrease in signal intensity of the additional bands. Interestingly, the additional bands under non-crosslinking conditions appear to be of different molecular sizes than the ones seen under crosslinking conditions (top, compare *lane 5* with *lanes 6 or 7*). It is not clear why this happens. In other experiments the additional bands are of the same size for all conditions. One explanation could be that at least part of the crosslinked protein complexes had a modified separation pattern during the SDS-PAGE. This could also indicate that, at least in this experiment, the crosslinking was specific to Yih1. Presumably, some interactions were preserved under crosslinking conditions but were lost under non-crosslinking conditions and this would result in a different migration pattern. No additional bands were detected on the western blot for GST alone overexpression samples (top, *lanes 1 to 4*). This could be due to a lower expression level than GST-Yih1. No additional bands were seen on the scanned image of the Coomassie stained gel (Figure C10, *bottom*), although a few were seen for GST-Yih1 by eye on the original gel.

After confirming the precipitation of GST proteins the presence of co-precipitating proteins was tested. Gcn1 is a Yih1-binding protein and it was found specifically in a complex with GST-Yih1 (Figure C10 middle, *lanes 1-4* vs. *lanes 5-8*). While GST alone also pulled down Gcn1 the signal was weaker in comparison to GST-Yih1-containing samples (e.g. *lane 1 vs*. *lane 5*). Additionally,

the signal intensity of Gcn1 followed the same pattern as the GST-Yih1 signal, i.e. there was a negative correlation with formaldehyde concentration.

If formaldehyde has stabilised the Yih1-Gcn1 complex then one would expect to see this complex as a band of high molecular weight on either the anti-GST or the anti-Gcn1 western blot. This band should be seen above the Gcn1 signal, near the top of the membrane, because of the large size of Gcn1 (297 kDa). However, no such band is visible. It is possible that the amount of complex formation was insufficient for detection because, while Yih1 is overexpressed, the cellular amount of Gcn1 was too low at about 7,300 molecules per cell (Ghaemmaghami et al., 2003). It is also possible that the additional bands were composed of different sets of proteins which would result in the detected migration pattern. Theoretically, the protein complexes that formed additional bands at a higher molecular weight could consist of GST-Yih1 plus other Yih1-binding proteins or of GST-Yih1 and Gcn1 degradation products. It is also possible that the complex had a different migration pattern due to the effects of formaldehyde crosslinking. In turn, a putative Gcn1-Yih1 complex might migrate similarly to monomeric Gcn1 because GST-Yih1 is relatively small at circa 64 kDa as compared to the 297 kDa large Gcn1 and this was not detected in this western blot.

Concluding from the above optimisation experiments, 0.3% formaldehyde was used because this concentration shows a reasonable compromise between protein loss and the formation of additional bands. The following outlines the preparation of samples for the protein identification via mass spectrometric analysis. The first trial involves crosslinking and non-crosslinking conditions and the presence of additional proteins such as actin (a YBP) and Pgk1 (a protein that does not bind Yih1) was tested.

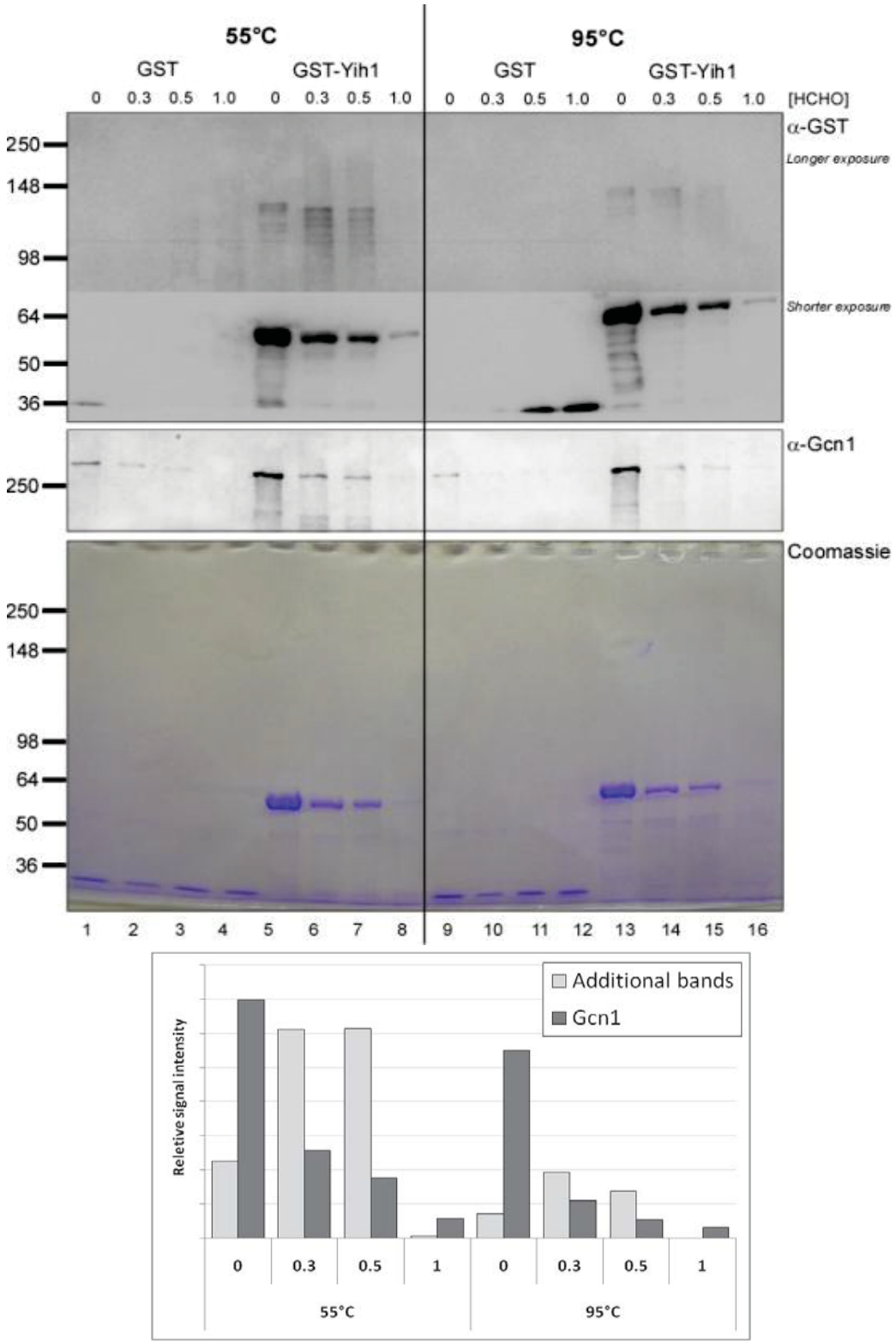


Figure C10: Formaldehyde crosslinking of yeast whole cell extract containing overexpressed GST-Yih1 and GST alone results in the formation of additional bands at higher molecular weight which partly disappeared after boiling of the samples at 95°C. A GST pulldown was performed and western blots to test the presence of GST proteins (*top*; two different exposure times) and co-precipitating Gcn1 (*middle*) were performed. No additional bands were detected on the Coomassie stained gel (*bottom*) but a few were seen for GST-Yih1 by eye on the original gel. Signal intensity for “Additional bands” was calculated as in Figure C8.

C.2.6 Protein identification Trial 1 using GST-Yih1 and GST was not successful

The result of one representative GST affinity purification is shown in Figure C11. GST and GST-Yih1 were pulled down specifically. Gcn1 co-precipitated specifically with GST-Yih1 but not GST.

Unexpectedly, while actin was expressed and detected in the input control, no actin co-precipitated. Moreover, the observation that no actin was detected under both crosslinking and non-crosslinking conditions suggested that the formaldehyde did not stabilise and retain protein-protein interactions, at least in this case for Yih1 and actin. It is unlikely that actin was lost during the whole cell extract preparation step because an actin signal is present in the input control (Figure C11). Instead, a significant portion of actin may have been lost either during the pre-adsorption step or during the washing steps after the pulldown (see Chapter B.13). Additionally, it was possible that the strong actin signal in the input control caused a rapid depletion of the detection substrate. Therefore, if the action concentration in the pulldown samples was low in comparison to the input then the substrate may have been used up before it was able to bind the antibody-actin complex in the pulldown samples and create a detectable signal.

Pgk1 is a highly expressed protein inside the cell with circa 314,000 molecules per cell (Ghaemmaghami et al., 2003). It was used as an indicator of unspecific binding of proteins because it is not known to interact with Yih1 or Gcn1. Pgk1 was detected in the input control but not in the pulldown samples, indicating that the level of unspecific binding was low.

In this experiment, one half of the pulldown sample was used for the SDS-PAGE and western blotting. The other half was used for the identification of proteins using mass spectrometry. For this, GST-Yih1 (and GST) and co-purifying proteins were eluted from the beads by glutathione in excess that leads to a competitive displacement of the GST proteins from the beads. In total, the elution of proteins was done three times for each sample. As seen in one representative western blot example GST-Yih1 and GST were successfully eluted (Figure C12, *lanes 1-12*). For each elution step a small reduction in signal intensity was seen, indicating that not all GST proteins may have been eluted. In fact, the beads that were subjected to all three elution steps still bound a relatively large number of GST proteins (*lanes 13-16*). The volume of the elution samples that were loaded

into the gel was 50% of the volume of the samples after the elution. Therefore, the signal intensity for the remaining proteins was slightly overrepresented and the detected signal should be half as strong. Nonetheless, not all proteins were eluted and it might be that the elution itself was not optimally efficient. In contrast to GST proteins the Gcn1 protein concentration in each elution step was markedly reduced and no Gcn1 was detected in the third elution (*lanes 11 and 12*), suggesting that all Gcn1 proteins were eluted. However, this was not the case and similarly to GST proteins, a significant amount of Gcn1 was not eluted (*lanes 15 and 16*). This supported the argument that the elution itself was inefficient. Pgk1 was detected in the input control but not in the elution samples and this indicated that the level of unspecific binding was low. The four samples of Elution 1 were submitted for protein identification to Auckland University. However, they did not identify any proteins, as the protein concentration was below the detection limit.

C.2.7 Formaldehyde crosslinking of His_6-Yih1 negatively affected the concentration of co-precipitated proteins

The GST tag is relatively large and could affect protein interactions. In addition, it dimerises. Therefore, another tag that is not known for dimerisation was tested for suitability for formaldehyde crosslinking and for the stabilisation of a Yih1-containing protein complex by formaldehyde. The tag hexahistidine (His_6) was used. His_6-Yih1 was overexpressed and the effect of formaldehyde on the formation of slowly migrating protein complexes was analysed.

Under crosslinking conditions His_6-Yih1 appeared as a strong signal between 36 and 50 kDa, corresponding to the expected size of ~45 kDa for this construct (Figure C13, *lanes 5-8* and *lanes 11-14*) (Sattlegger et al., 2011). The His antibody detected no signal in the vector-alone samples, as expected (*lanes 1-4* and *lanes 9-10*). For the samples containing 50 µg of protein slowly migrating additional bands were present only under crosslinking conditions (*lane 6*). They disappeared with boiling of the samples (*lane 8*), indicating that the additional bands are due to formaldehyde crosslinking. in contrast to the GST tag experiments above a lower number of additional bands were detected.

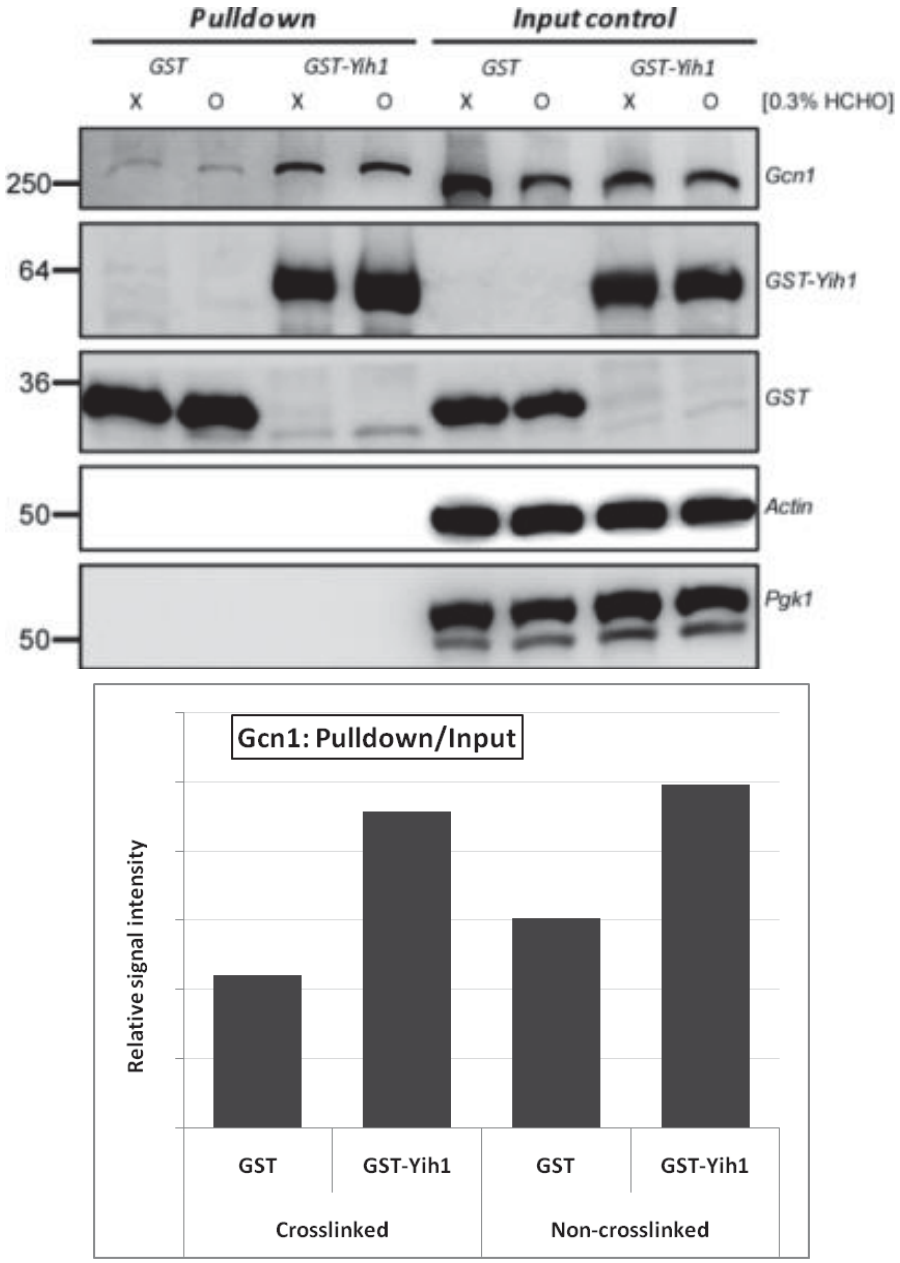


Figure C11: Gcn1 specifically co-precipitates with GST-Yih1 in a GST pulldown while actin and Pgk1 did not. Formaldehyde crosslinking of yeast whole cell extract, followed by GST pulldown and elutions. Western blots using antibodies against Gcn1, GST, actin and Pgk1 were done. X: Crosslinked. O: Non-crosslinked. For Gcn1 the signal ratios of pulldown to input control are shown in the graph at the bottom.

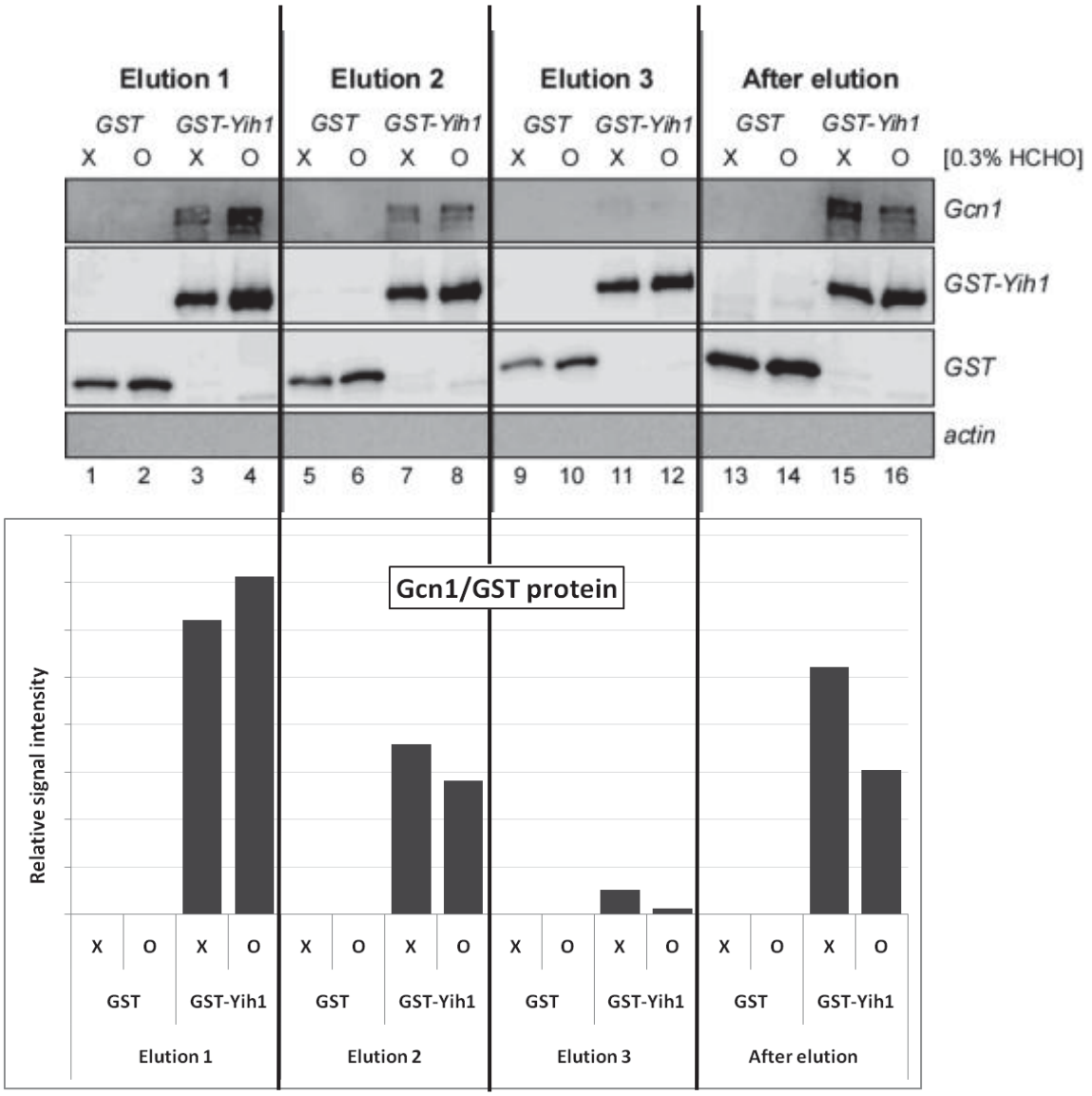


Figure C12: Gcn1 and the GST proteins were successfully eluted from the beads. Actin is not detectable, as expected from the GST pulldown above. Formaldehyde crosslinking of yeast whole cell extract, followed by GST pulldown and elutions. Western blots using antibodies against Gcn1, GST and actin were done. Each sample was eluted three times. "After elution" refers the amount of protein left on the beads after all three elutions and the volume loaded is twice the volume of each elution step. For Gcn1 the signal ratios of Gcn1 to GST protein are shown in the graph at the bottom. The values for "after elution" were reduced by 50% to take into account that the loaded volume was twice of the "elution" lanes.

The signal for His_6-Yih1 was relatively strong compared to the signal for the additional bands, suggesting that the majority of His_6-Yih1 was not in a crosslinked complex or that HCHO did not crosslink all proteins.

Doubling the total protein amount of the whole cell extract to 100 µg resulted in a smear on the anti-His western blot under crosslinking conditions but not under non-crosslinking conditions (*lanes 12*). The smear indicates that the proteins did not separate well during electrophoresis and that this was due to the formaldehyde crosslinking. Additionally, this smear did not disappear when boiling these samples at 95˚C (*lane 14*), indicating that 100 µg of total protein is too much to study formaldehyde crosslinking.

His_6-Yih1 was then used for affinity purification experiments. For these, Yih1 with a mutation in helix 2 (His_6-Yih1*H2) was included because it showed an increased binding affinity to other proteins (Sattlegger et al., 2011). After the pulldown the western blot was probed for a number of proteins, as in the GST pulldown experiments above. The result is shown in Figure C14. His_6-Yih1 was successfully pulled down while the vector control did not show any signal, as expected. There was a smear for His_6-Yih1 and 0.5% formaldehyde (*lane 8*) but this has not affected the pulldown signal (*lane 4*). Gcn1 co-precipitated specifically with both Yih1 types only under non-crosslinking conditions (*lanes 3 and 5*). For His_6-Yih1 this could be explained be either insufficient amounts of Gcn1 or too much, as indicated by the smear in the input for Gcn1 (*lanes 8 and 10*). However, this was not the case for the Yih1 mutant where a Gcn1 signal and no smear was detected (*lane 12*).

In addition to these proteins the presence of elongation and initiation factors was tested. They are known to play a role in Gcn2 regulation (Visweswaraiah et al., 2011b, 2012) and preliminary studies found that they might bind to Yih1 (Evelyn Sattlegger, unpublished results). Elongation factors eEF1A and eEF3 and initiation factor Gcd6 (eIF2B subunit ε) were co-precipitating with Yih1. However, both proteins were detected in the vector-containing samples and this indicated unspecific binding to the beads. Exposure to formaldehyde negatively affected protein concentration which resulted in a smear or no protein (*lanes 8 and 10*, respectively) and subsequently in weak or no precipitation of proteins in the pulldown.

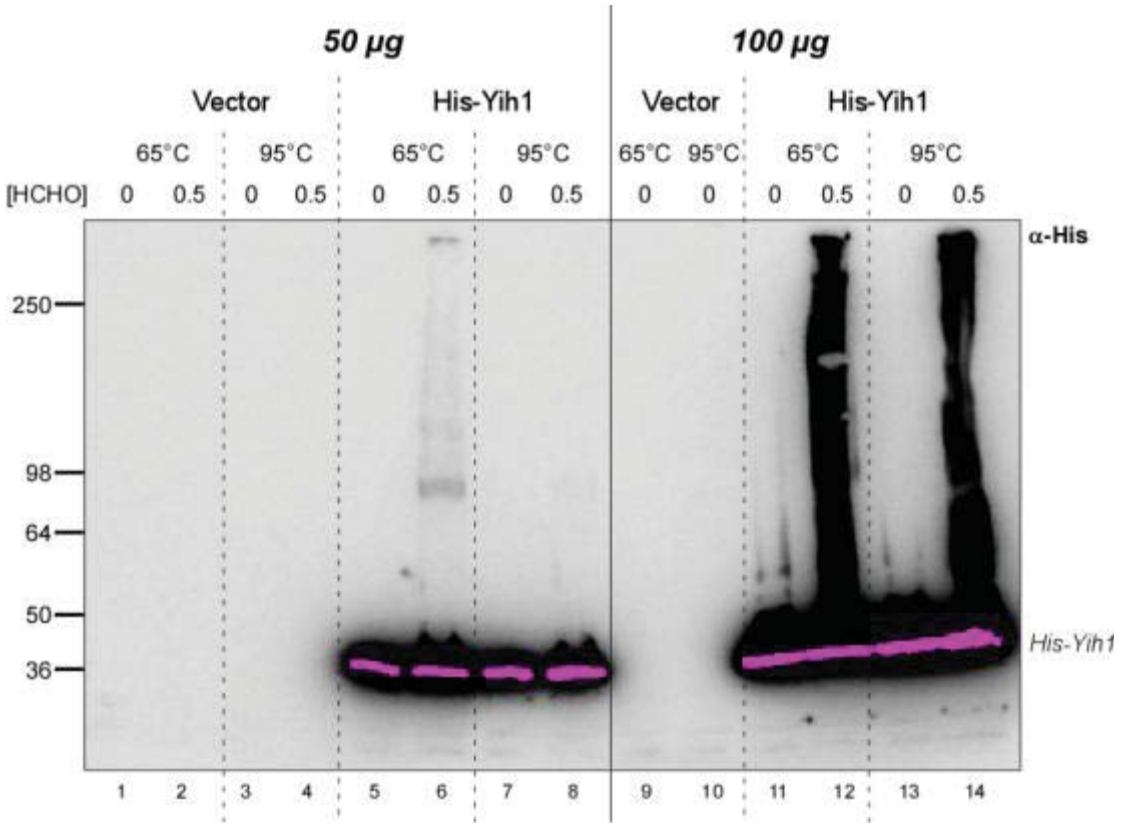


Figure C13: Formaldehyde crosslinking of yeast whole cell extract containing overexpressed His_6-Yih1 results in relatively weak additional bands compared to the GST-Yih1 experiments. These bands fully disappear after boiling when 50 µg was loaded but only very little when 100 µg of protein extract was used. Proteins were visualised using antibodies against the His epitope. Note: In this experiment the samples of 0.5% for the vector with 100 µg are missing because of insufficient amount of samples.

Actin was not pulled down (the band seen in lane 3 was background signal from another western blot), similar to the GST affinity purification in Figure C11.

Initial experiments by M. Dautel, D. Amberg and E. Sattlegger provided evidence that Cdc28 might be in a complex with Yih1. This protein co-precipitated with Yih1 but a signal was detected for vector-containing samples as well (Figure C14, *lanes 3 and 5 and lane 1*, respectively). No Cdc28 signal was detected for crosslinked pulldown samples although there was an input signal for crosslinked vector and Yih1 mutant that was equal to non crosslinked samples.

Pgk1 as an indicator for unspecific binding was not pulled down (Figure C14).

For His-Yih1*H2 the input control samples were of similar intensity under crosslinking and non-crosslinking conditions but for the pulldown samples the protein concentration for crosslinked conditions was lower. This effect was even

more pronounced for Gcn1: The Gcn1 signal in His-Yih1*H2-expressing samples was stronger for formaldehyde-treated cells while almost no Gcn1 co-precipitated. It was difficult to determine if this was the case for GST-Yih1 as well due to the smear. However, it was possible that crosslinking might prevent efficient affinity purification.

Taken together, the results for GST-Yih1 and His-Yih1 indicated that using formaldehyde does not have a distinct advantage over non-crosslinking conditions for Yih1-facilitated YBP precipitation. In fact, crosslinking repeatedly resulted in the loss of proteins, possibly due to the formation of insoluble protein complexes that were lost during the cell lysis and purification procedure. Additionally, there was evidence that formaldehyde can negatively affect the co-precipitation of the known YBP Gcn1 with Yih1. Furthermore, it cannot be confirmed if formaldehyde stabilised interactions between Yih1 and other proteins. The first amino acids that are crosslinked are cysteine, lysine and tryptophan (Toews et al., 2008). Thus, it may be that these amino acids are less accessible in Yih1 or not present in sufficient amounts in Yih1 or its interaction partners. Supporting the latter idea, there are ten lysine and only four tryptophan and four cysteine residues in Yih1 (out of 258 amino acids in total). Therefore, no formaldehyde crosslinking was used to identify potential YBP in this study and non-crosslinking conditions were applied instead.

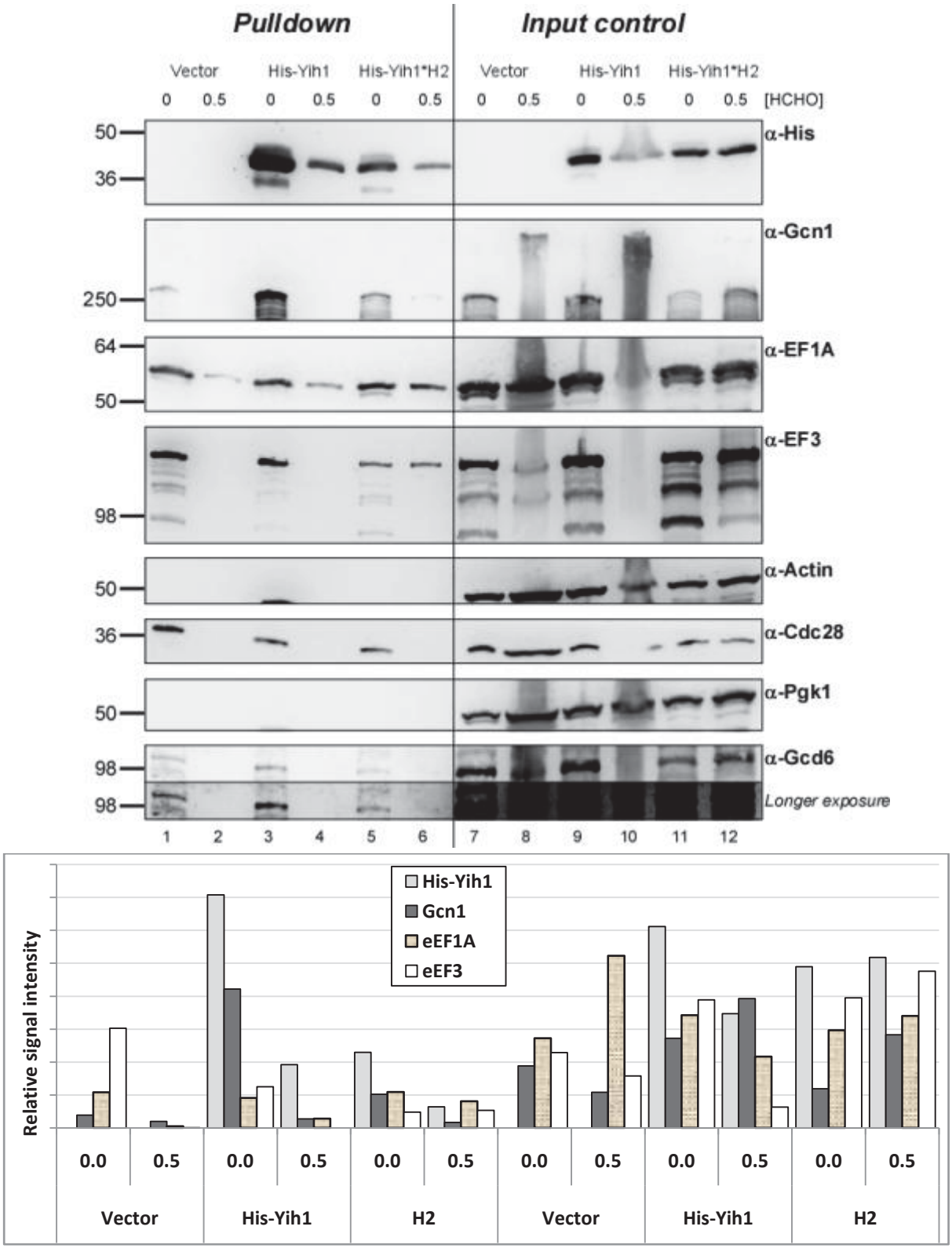


Figure C14: Formaldehyde crosslinking of yeast whole cell extract containing overexpressed His_6-Yih1 and His_6-Yih1*H2, followed by a His pulldown, specifically co-precipitates Gcn1. eEF1A, eEF3, Cdc28, and Gcd6 co-precipitate with both His-Yih1 and the vector alone control. Actin, Gcn2, and Pgk1 are not co-precipitating with Yih1 or the vector control. Western blots using antibodies against His, Gcn1, eEF1A, eEF3, Cdc28, Pgk1 and Gcd6 were done.

C.2.8 Protein identification Trial 2 using GST-Yih1 and GST-Yih1*H2

Elution was inefficient in Trial 1 and under non-crosslinking conditions the background binding was still too high. Therefore, an independent pulldown experiment was conducted by Evelyn Sattlegger. In addition, she tested for the presence of GST proteins, Gcn1 and actin in a western blot and she performed a Ponceau staining.

GST-Yih1 specifically co-precipitated Gcn1 and actin (Figure C15 and Figure C16). For this reason the pulldown samples of this experiment were used for identifying protein co-precipitating with Yih1. Samples were separated via SDS-PAGE, subjected to Coomassie staining and then submitted for mass spectrometry. The other half of each sample was used for western blotting. In addition, Yih1 with mutations in helix 2 (GST-Yih1*H2) was included because it has a higher binding affinity in general as well as a C-terminal Yih1 fragment (GST-Yih1[68-258]) because it showed strong binding to actin and Gcn1 (Sattlegger et al., 2011). No crosslinking was used because the previous results were not convincing enough in regards to the ability of formaldehyde to stabilise protein-protein interactions.

Ponceau S staining showed the successful pulldown of GST-Yih1, GST-Yih1*H2 as well as GST alone (Figure C15). The Yih1 fragment GST-Yih1(68-258) was not detected, probably because its expression levels was lower compared to full length Yih1 and the Yih1 mutant (Sattlegger et al., 2011).

All four GST proteins variants were pulled down successfully, as seen in western blotting (Figure C15, *lanes 1-6*). While the Yih1 fragment was not detected on the Ponceau S it was seen when using GST antibodies (*lane 2*). While the expression level of the fragment was less than half of GST-Yih1 in this western blot its expression level appeared much lower in previous studies (Sattlegger et al., 2011).

Gcn1 co-precipitated specifically with GST-Yih1, GST-Yih1(68-258) and GST-Yih1*H2. Curiously, no Gcn1 was detected in the input control. Shown here is only the part of the membrane where Gcn1 was expected. Looking at the whole anti-Gcn1 western blot membrane revealed a large number of bands at molecular weights below 148 kDa, indicating protein degradation. This degradation signal was also seen for the pulldown samples, although to a lower extent. The Gcn1

signal intensity for the Yih1 fragment-expressing samples was similar to the signal seen in the GST-containing sample, indicating it did not co-precipitate Gcn1 specifically (*lanes 2 vs. lanes 4 and 5*). This is in contrast to previous research that showed strong Gcn1-binding to the C-terminus of Yih1 and that it needed the N-terminus of Yih1 for its interaction (Sattlegger et al., 2011).

In this experiment actin was pulled down specifically with all of the Yih1 proteins although the actin signal for GST-Yih1-containing samples is relatively weak compared to the Yih1 mutant.

eEF1A and eEF3 co-precipitated with Yih1*H2 specifically as compared to GST alone (*lane 3* vs. *lanes 4 and 5*). Gcd6 also co-precipitated with Yih1*H2. Pulldown samples containing GST-Yih1 (*lane 1*) and the Yih1 fragment (*lane 2*) show a barely detectable signal for either eEF3 or Gcd6 and thus relatively weak precipitation compared to the Yih1 H2 mutant. While the same is true for eEF1A it appeared to show a specific association with full length Yih1 compared to GST alone (*lane 1* vs. *lanes 4 and 5*). This suggested that eEF3 and Gcd6 were not co-precipitating specifically with GST-Yih1 or the Yih1 fragment.

Cdc28 was detected in the input control but not in the pulldown, indicating that at least in this experiment it did not co-precipitate with Yih1 or that the amounts that co-precipitated were too low for detection. Pgk1 was not pulled down and this indicated a low level of unspecific binding (it needs to be noted that the overall signal is weak and therefore weak or unspecific binding may not be detected). Finally, Gcn20, a Gcn1-binding protein, might co-precipitate with Yih1 via Gcn1 but no signal was seen, maybe due to a weak antibody.

The signal that was detected on the membrane in Figure C15 is likely an eEF3 background because it appeared at the same molecular weight and showed the same shapes. This background appeared because the membrane was first exposed to eEF3A antibodies and then Gcn20.

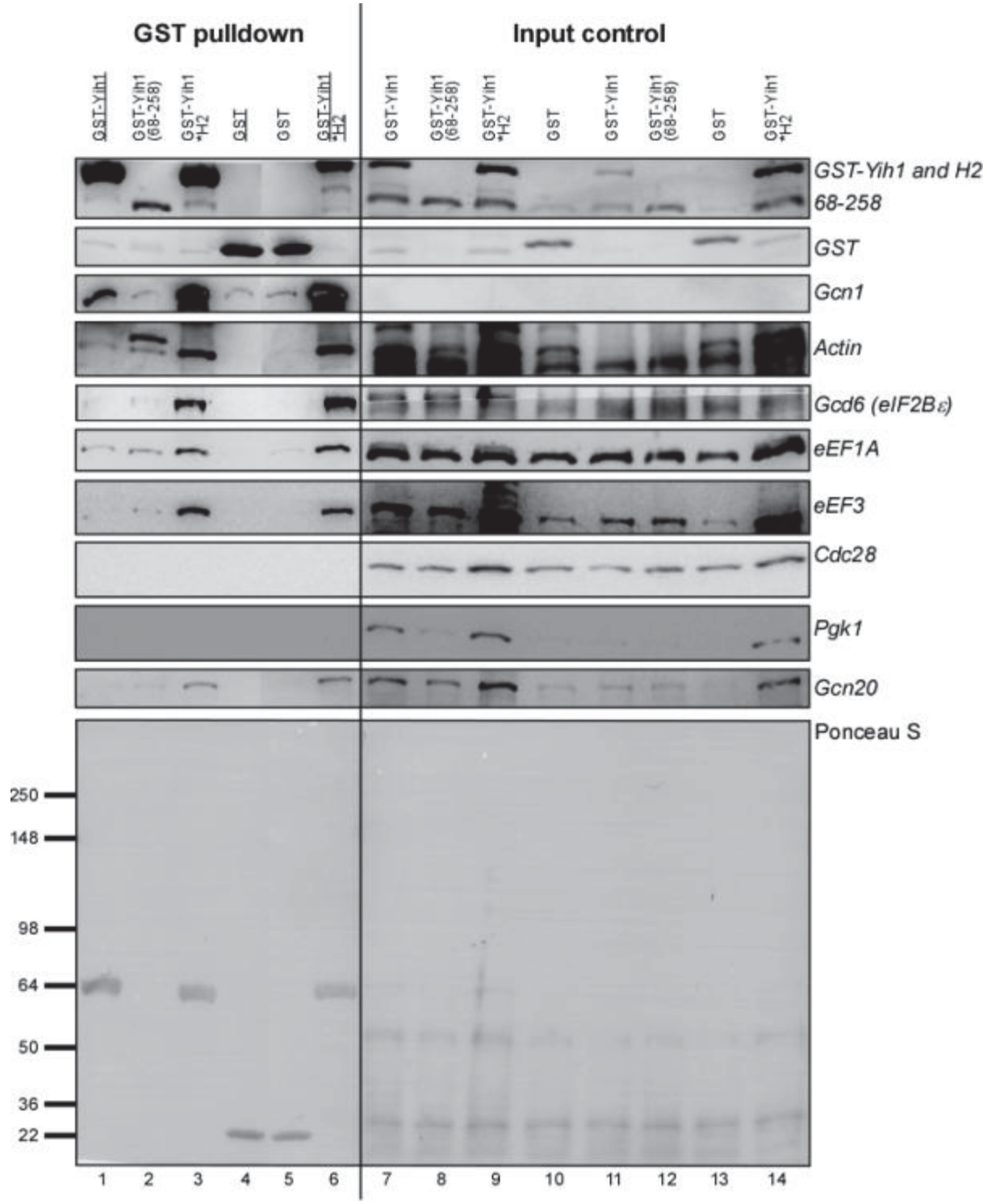


Figure C15: Gcn1 and actin specifically co-precipitated with GST-Yih1as well as the Yih1 fragment GST-Yih1(68-258), and the Yih1 mutant GST-Yih1*H2 in a GST pulldown without crosslinking. Gcd6, eEF1A, EF3, and Gcn20 co-precipitated strongly with GST-Yih1*H2. Ponceau S staining showed the successful pulldown of GST-Yih1, GST-Yih1*H2 as well as GST alone but the Yih1 fragment GST-Yih1(68-258) was not detected. No formaldehyde was used. Western blots using antibodies against GST, Gcn1, actin, Gcd6, eEF1A, eEF3, Cdc28, Pgk1 and Gcn20 were done. Underlined: Samples used for mass spectrometry (Figure C17). Quantification of the western blot is shown in Figure C16.

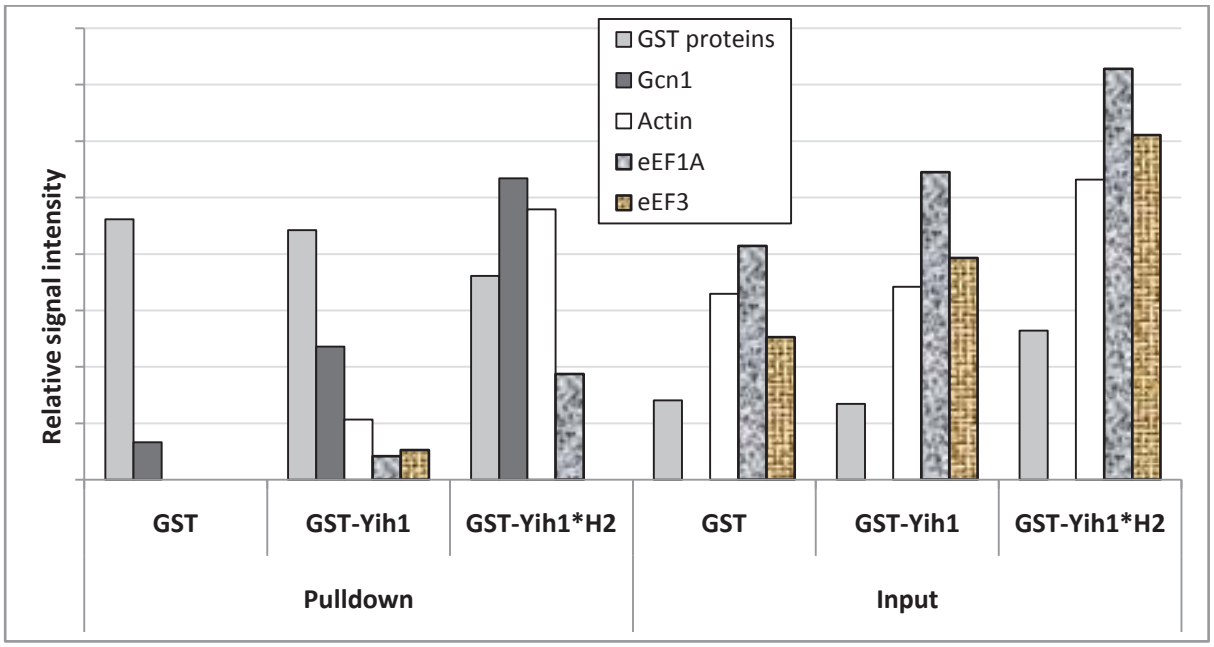


Figure C16: Quantification of western blot in Figure C15.

Of all the Yih1 proteins that were used Yih1*H2-containing samples showed a strong pulldown signal for most of the proteins tested. This indicated a strong association with other proteins, confirming earlier experiments where it showed a higher binding affinity to Gcn1 and actin than full length Yih1 (Sattlegger et al., 2011). Additionally, the findings that Yih1*H2 precipitated a relatively large amount of Gcd6 and eEF3, even though they were not specifically precipitated by GST-Yih1 or the fragment, give rise to the possibility that the mutations in Yih1 resulted in increased level of unspecific interactions. Accordingly, this needs to be taken into account when using Yih1*H2.

All GST proteins including GST alone were expressed at different levels, according to the signal intensities of their respective bands in the input control. This was seen in earlier experiments (Sattlegger et al., 2011). This could affect judgments about the specificity of co-precipitating proteins. In the input control, the GST alone signal intensity was less than half than the signal intensities of GST-Yih1 and the Yih1 mutant (*lanes 10 and 13* vs. *lanes 7, 9 and 14*). The Yih1 fragment appeared to be expressed at similar levels as GST (*lanes 8 and 12*). Taking this into account the Gcn1 co-precipitation remained significant for both GST-Yih1 and the mutant (e.g. *lane 1* vs. *lane 4*). GST-Yih1[68-258] still co-precipitated actin specifically (*lane 2* vs. *lane 4*). For the other proteins their co-precipitations with the Yih1 mutant remained significant, including eEF1A

precipitation by GST-Yih1 (*lane 1* vs. *lane 4*). The amount of actin that was pulled down by GST-Yih1 is relatively low. This did not allow a definite conclusion on the specificity after taking into account the expression level of GST-Yih1 and GST. This did not affect any conclusions in regards to the precipitation of Gcd6 and eEF3 by GST-Yih1 because these results were not significant in the first place.

To summarise, in this experiment Yih1 successfully co-precipitated Gcn1 and to some extent actin. The Yih1*H2 mutant showed strong precipitation of most of the proteins tested.

In preparation for the mass spectrometry pulldown, samples containing GST-Yih1, GST-Yih1*H2 and GST alone were run on an SDS-PAGE and stained with Coomassie blue to visualise the protein bands (Figure C17). The Yih1 fragment was not included. For both GST-Yih1 and GST-Yih1*H2 two distinct bands were detected: The lower one at around 64 kDa was corresponding to the overexpressed Yih1 because it was running at the expected molecular weight (Figure C17). The top band at around 250 kDa was of weaker intensity. The areas containing the GST-Yih1 (and GST alone) were removed and analysed separately. This was done because the strong GST signal might, during mass spectrometry, mask the signal of other proteins that are not as abundant. For the same reason, the additional band at the top was analysed separately as well. The remaining areas of the gel was “pooled” into one fraction. The analysis was done at the Centre for Protein Research, University of Otago. For the result analysis below the data from the removed areas and the pooled samples were compiled into one dataset.

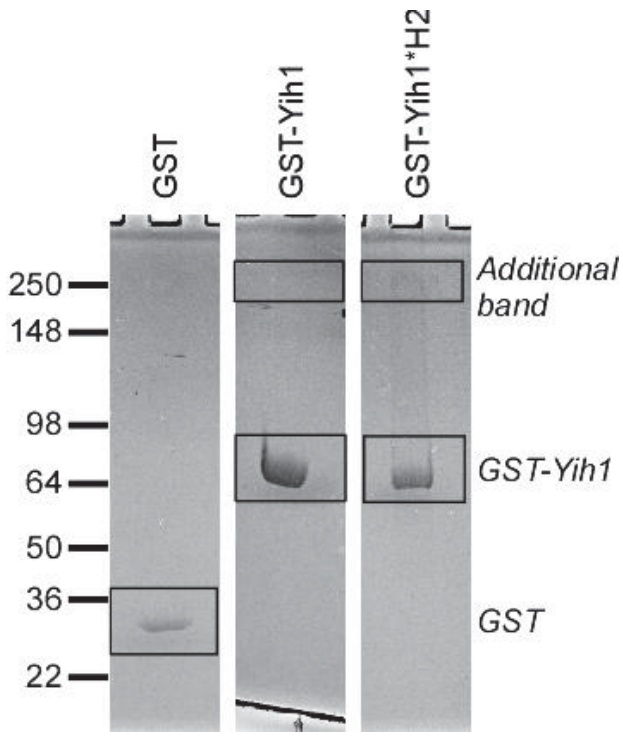


Figure C17**:** Overview of the three lanes used for mass spectrometry analysis of a Coomassie-stained SDS-PAGE gel. Black rectangles indicate areas that were removed and analysed separately. The remaining gel was analysed as one.

C.2.9 Gcn1, actin and eEF1A were identified in the in-house experiments

In total, 107 proteins were identified as co-precipitators of GST-Yih1 and 288 for GST-Yih1*H2. A large number of proteins (188) were unique to Yih1*H2 samples while only 7 were unique to GST-Yih1 (Figure C18 and Table C4). This resulted in an overlap of 100 proteins between the two different types of Yih1. In total 295 unique proteins were identified and all of them were considered significant. The higher number of co-precipitating proteins for the Yih1 mutant could be a result of its higher binding affinity to other proteins: If we assume that the Yih1 mutant has a higher affinity to other proteins then we would expect it to pulldown not only a larger variety of proteins but also a larger amount of each protein. Thus, it is more likely that the amounts of peptides that were available for protein identification are above the significance threshold. Presumably, this was not the case for GST-Yih1 and a larger number of proteins were excluded, reducing the total number of identified proteins.

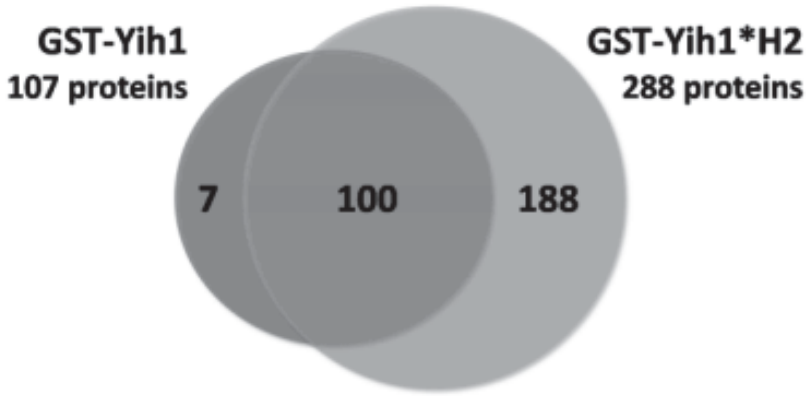


Figure C18: Number of protein that co-precipitated with GST-Yih1 and GST-Yih1*H2. Most of the proteins that co-precipitated with GST-Yih1 also co-precipitated with GST-Yih1*H2 in the mass spectrometry analysis. See Table C4 for the proteins.

Gcn1 was only found to co-precipitate with GST-Yih1*H2. As seen above in the western blot the Gcn1 signal was weaker for GST-Yih1 than for Yih1*H2 (cf. Figure C15 and Figure C16). Only Yih1*H2 precipitated a sufficient amount of Gcn1 for identification. One reason why Gcn1 was not seen in the mass spectrometry analysis is that western blotting can be more sensitive. This was also the case for Gcn2 and Gcd6 which were identified in the western blot for GST-Yih1*H2 but not in the mass spectrometry (Table C5, *column "WB" and "MS"*, respectively). eEF1A and eEF3 were identified with both GST-Yih1 and GST-Yih1*H2. eEF3 is unexpected because it was not seen on the western blot. This could indicate that, in this case, the western blot was less sensitive than mass spectrometry in regards to protein identification. Neither Cdc28 nor Gcn20 were found as co-precipitators of either GST-Yih1 or GST-Yih1*H2. For Cdc28 this was seen in the western blot result, suggesting that it did not form a complex with Yih1 in this experiment. Unexpectedly, Pgk1 was found with GST-Yih1*H2 even though the western blot did not show any signal for the pulldown samples. This again suggested an increased binding affinity of the Yih1 H2 mutant.

Table C4: Full list of all proteins that were identified to be in a complex with GST-Yih1 or GST-Yih1*H2 in the mass spectrometry based on in-house experiments. Proteins that were found in both data sets are shaded grey. Gcn1 and actin are in bold.

GST-Yih1		
Acc1	Kgd2	RPL24A
Act1	Leu2	RPL28
Ade3	Lpd1	RPL35A
Adh1	Lys1	RPP0
Aha1	Lys12	RPS0A
Atp1	Lys20	RPS1B
Atp2	Mdj1	RPS2
Atp4	Met10	RPS3
Bmh2	Met17	RPS14A
Cit1	Mir1	Rvb1
Coq1	Nde1	Rvb2
Cor1	Ndi1	Sam1
Cyt1	Nfs1	Shm2
Dbp5	Nop58	Ssa1
Ded1	Pet9	Ssb1
Eft1	Pfk1	Ssc1
Egd2	Pma1	Sub2
Eno2	Por1	Tdh3
Faa1	Psa1	Tef1
Faa4	Qcr2	Tif1
Fas1	Rnr1	Trp5
Fas2	RPL1A	Tub2
Gal1	RPL2A	Ugp1
Gdh1	RPL3	Ura2
Gnd1	RPL4A	Ura3
Gus1	RPL4B	Ura7
Gvp36	RPL7A	Vma2
Hsc82	RPL8A	Vma5
Hsp104	RPL10	Vps1
Hsp82	RPL11A	Ydj1
Idh1	RPL12A	Yef3
Idh2	RPL13B	Yhb1
Idp1	RPL17A	Yhm2
Ilv2	RPL19A	
Imd3	RPL20A	
Kap123	RPL21A	
Kgd1	RPL23A	

GST-Yih1*H2					
Abp1	Eht1	Lys12	Ret2	RPS5	Sxm1
Acc1	Eno2	Lys20	Rna1	RPS6A	Tcp1
Ach1	Erg6	Lys4	Rnr1	RPS7A	Tdh1
Aco1	Ett1	Mcm6	Rpa49	RPS7B	Tdh2
Acs2	Faa1	Mcm7	Rpa190	RPS8A	Tdh3
Act1	Faa4	Mcr1	Rpb2	RPS9B	Tef1
Ade3	Fas1	Mdh3	Rpc40	RPS11A	Tef4
Ade5,7	Fas2	Mdm38	Rpg1	RPS13	Tif1
Aha1	Fks1	Mdn1	RPL1A	RPS14A	Tif34
Ald4	Gal1	Met3	RPL2A	RPS16A	Tif35
Alo1	Gal7	Met5	RPL3	RPS18A	Tim44
Arf1	Gal10	Met10	RPL4A	RPS20	Tpd3
Arg5,6	Gal80	Met17	RPL4B	RPS22A	Trp5
Aro1	Gcn1	Met18	RPL5	RPS23A	Tub1
Atp1	Gcs1	Mir1	RPL6A	RPS24A	Tub2
Atp2	Gdh1	Mpm1	RPL7A	RPS26A	Tub3
Atp3	Gfa1	Mrp13	RPL8A	Rpt2	Tuf1
Atp4	Glt1	Mvp1	RPL8B	Rpt3	Ubi4
Ayr1	Gnd1	Myo2	RPL9A	Rpt4	Ubr1
Bbc1	Gpm1	Nde1	RPL10	Rpt5	Ugp1
Bem2	Grx3	Ndi1	RPL11A	Rpt6	Ura2
Bmh1	Gsy2	Nfs1	RPL12A	Rtn1	Ura3
Bmh2	Gus1	Nip1	RPL13A	Rvb1	Ura7
Cbr1	Gut2	Nop1	RPL14A	Rvb2	Utp10
Cct3	Gvp36	Nop56	RPL15A	Rvs167	Vip1
Cct4	Hem15	Nop58	RPL16A	Sac6	Vma1
Cct6	Hgh1	Ola1	RPL16B	Sam1	Vma2
Cct7	His5	Osh2	RPL17A	Sam2	Vma5
Cct8	Hog1	Pab1	RPL18A	Sar1	Vma8
Cdc19	Hom3	Pda1	RPL19A	Sdh1	Vma13
Cdc48	Hsc82	Pdb1	RPL20A	Sds24	Vps1
Chc1	Hsp104	Pdc1	RPL21A	Sec21	Ycp4
Cit1	Hsp78	Pet10	RPL23A	Sec26	Ydj1
Clu1	Hsp82	Pet9	RPL24A	Sec27	Yef3
Cop1	Idh1	Pfk1	RPL25	Sec4	Yhb1
Coq1	Idh2	Pfk2	RPL26A	Sec7	YHR033W
Cor1	Idp1	Pgk1	RPL27A	Ser1	YKR018C
Cpa2	Ilv1	Pil1	RPL28	Shm2	Ykt6
Cpr6	Ilv2	Pim1	RPL31A	Sis1	Yop1
Crm1	Imd3	Pma1	RPL33A	Sla2	Ypk1
Cse1	Iml2	Por1	Rpn2	Srp1	Ypt1
Csr1	Kap123	Pre10	Rpn7	Srv2	Zpr1
Dbp5	Kap95	Pre6	RPP0	Ssa1	Zwf1
Ded1	Kgd1	Psa1	RPS0A	Ssa2	
Dnm1	Kgd2	Pse1	RPS1A	Ssb2	
Dpm1	Leu2	Pst2	RPS1B	Ssc1	
Ecm33	Lhs1	Qcr2	RPS2	Stm1	
Eft1	Lpd1	Rbg2	RPS3	Sub2	
Egd2	Lys1	Rep1	RPS4A	Sup45	

Actin was identified as a co-precipitating protein for both GST-Yih1 and GST-Yih1*H2 (Table C5, *column "MS"*). This result was in agreement with the western blot and with previous studies (Figure C15) (Sattlegger et al., 2004). This showed that, even though the signal intensity for actin in the western blot was relatively low, there was enough protein available for protein identification.

For the samples that expressed GST alone only GST itself was identified while no proteins were found in the pooled fraction. Although theoretically no other proteins should be identified there is always a certain level of unspecific interactions. The amount of unspecific interactors presumably was below the significance threshold and thus they were not identified. Gcn1, for example, was seen in the western blot in GST-containing samples as a relatively weak signal that indicated a certain amount of unspecific binding but its level presumably was too low for identification in the mass spectrometry. This was important to consider in regards to eEF3: Since it was not identified with GST alone then this would support the notion that it is indeed in a complex with GST-Yih1. On the other hand, if a protein is not identified then this does not mean that there is no interaction with Yih1.

The Yih1 mutant showed a higher affinity to other proteins and this could be due to unspecific interactions. However, this does not exclude the possibility that this higher affinity allows the detection of specific but weak interactions that were lost when using the wild type Yih1. One important example is Gcn1 which was seen in both western blot and mass spectrometry only for the Yih1 mutant (Table C5).

GST-Yih1 and GST-Yih1*H2 were identified in the upper additional band that was cut from the gel, as seen in the non-compiled data (i.e. the set that was separated into three files as per Figure C17). These may be due to GST dimerisation or this may suggest that a portion of additional bands consist of a stable Yih1-YBP complex.

Table C5: GST pulldown of GST-Yih1, GST-Yih1*H2, and GST: Comparison of identified proteins in western blot (WB) and mass spectrometry (MS). Highlighted text indicates proteins that were detected in both western blot and mass spectrometry.

	GST-Yih1		GST-Yih1*H2		GST	
	WB	MS	WB	MS	WB	MS
Actin	yes	yes	yes	yes	no	no
Cdc28	no	no	no	no	no	no
Gcd6	no	no	yes	no	no	no
Gcn1	yes	no	yes	yes	no	no
Gcn20	no	no	no	no	no	no
eEF1A	yes	yes	yes	yes	no	no
eEF3	no	yes	yes	yes	no	no
Pgk1	no	no	no	yes	no	no

C.3 Discussion

Both the published and in-house dataset were combined to form the Yih1 interactome (see Appendix A for full list of proteins). As outlined above, based on Krogan et al. (2006) 1,803 potential YBP that were found in a complex with Yih1 were identified. Three additional potential binding proteins were identified by two other studies (Jsn1, Pir5 and Urh1), adding up to 1,806 published proteins that were in a complex with Yih1 at least once. Using the in-house approach 295 proteins were identified that were found in a complex with GST-Yih1 or GST-Yih1*H2 significantly. The proteins that were found with both approaches are given in Figure C19. Groups of proteins were organised as Sets, denoted by encircled roman numerals in Figure C19, in order of importance and with Set I being the most important in regards to novel YBP. In the following section, protein function and localisation data was taken from yeastgenome.org, unless otherwise noted (Cherry et al., 2012).

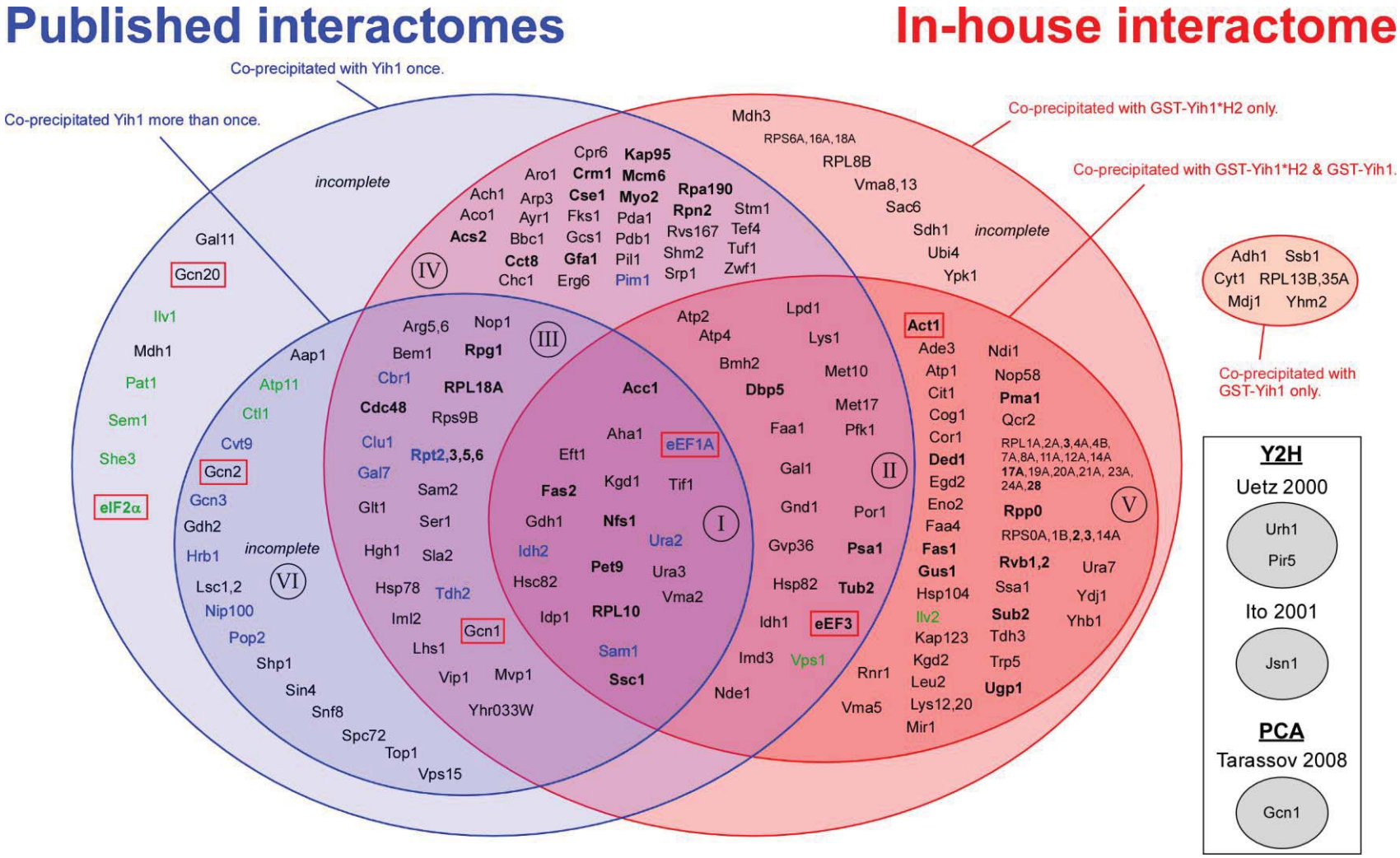


Figure C19: Overlap between published and in-house interactome. Blue: Proteins that are in complex with TAP-Yih1. Roman numbers refer to the Sets discussed in the text. Green: Tagged proteins that co-precipitated Yih1. Bold: Essential proteins. Red box indicates known GAAC proteins. Yeast-two hybrid (Y2H) and protein complementation assay (PCA) findings are given on the side. Incomplete: Indicates that only a protein selection is shown due to the large number of identified proteins in that set.

Set I contains potential YBP that were found more than once in a complex with Yih1 (either tagged or untagged) in the published interaction screens and that were also found in a complex with both GST-Yih1 and GST-Yih1*H2 in the in-house screen. Therefore, these proteins are the most likely candidates for novel Yih1-binding proteins. This set contains eEF1A, a protein known to be involved in the GAAC (Visweswaraiah et al., 2011b). eEF1A as well as Idh2, Ura2 and Sam1 were found in a complex with TAP-tagged Yih1 by Krogan et al. (2006) while TAP-Ilv2 co-precipitated Yih1 in the same study. Of those proteins only eEF1A was found with a high score in the published data, suggesting that this protein may be a YBP. However, this does not exclude the possibility that the low scoring proteins are YBP as well. Another protein of note in this set is Elongation Factor 2, Eft1, which is involved in ribosomal translocation during protein synthesis. Interestingly, Hsc82 is known to be involved in Gcn2 protein maturation (Donze and Picard, 1999). It belongs to the Hsp90 family of heat shock proteins that are involved in protein folding and that are activated under heat stress (Borkovich et al., 1989). Aha1 from this set is a co-chaperone that binds and activates Hsp90 proteins (Panaretou et al., 2002). Like Hsp90, it is needed for cell survival under stress conditions. Thus, these findings suggest a possible regulatory link between Gcn2 and Hsc82 that is mediated by Yih1. Strikingly, Idh2, Ilv2, Kgd1 and Pet9 are known to localise only to the mitochondrion, suggesting that Yih1 may be found in this organelle and suggesting novel functions for Yih1. The protein Vma2 is a subunit of the vacuolar H^+-ATPase and is involved in intracellular acid resistance (Kawahata et al., 2006; Lawrence et al., 2004), just as was found for Gcn2 (Hueso et al., 2012). Strains deleted for *VMA2* were found to be SM sensitive (Parsons et al., 2004), suggesting an involvement in Gcn2 regulation.

Set II is similar to Set I in that it contains potential YBP that were found in a complex with both GST-Yih1 and GST-Yih1*H2. The difference is that it contains potential YBP that were found only *once* in a complex with Yih1 in the published data. eEF3 is found in this set and, like eEF1A, is involved in the GAAC (Visweswaraiah et al., 2012). Hsp82, the other member of the Hsp90 family in yeast, belongs to this set and supports the idea of a link between Gcn2, Yih1 and Hsp90 family proteins. This set contains three proteins that are part of the cytoskeleton, Gvp36 and Vps1 (both involved in actin organisation) and Tub2 (a microtubule component), supporting the idea that there is a connection between

Gcn2, Yih1 and the cytoskeleton. Again, a number of proteins are localised to the mitochondrion. Among them is Idh1 which, together with Idh2 from Set I, are subunits of the isocitrate dehydrogenase that is involved in the ATP production in the tricarboxylic acid cycle (Cupp and McAlister-Henn, 1991). This provides further evidence for possible novel roles of Yih1 in the mitochondrion.

Set III is also similar to Set I but it contains proteins that were only found with GST-Yih1*H2, in addition to the published data. This set contains the known Yih1-binding protein Gcn1[2]. Five proteins were found in a complex with TAP-Yih1 (Cbr1, Clu1, Gal7, Rpt2 and Tdh2) of which Cbr1 and Tdh2 localise to the mitochondrion. Translation initiation factor eIF2 co-precipitated Yih1 in the published study by Krogan et al. (2006). As eIF2 has an important role in the GAAC and thus it is not surprising that another initiation factor was identified, namely subunits Clu1 and Rpg1 of eIF3. The essential proteins Rpt2, Rpt3, Rpt5 and Rpt6 are involved in proteasome function which could suggest that they are unspecific interactors. On the other hand Rpt5 has been found as a co-precipitator of Hsp82 which was identified in Set II (Guerrero et al., 2006). Furthermore, proteins are marked for degradation by the proteasome by being labelled with ubiquitin (Ubi4) (Lecker et al., 2006). However, in the Yih1 interactome ubiquitin was only identified as a co-precipitator of GST-Yih1*H2, even though it is a promiscuous protein and therefore its identification would be expected (Peng et al., 2003; Swaney et al., 2013). This could hint at a functional Yih1-proteasome relationship and this could involve Gcn2 (Lee 1998). This is supported by the observation that exposing mouse embryo fibroblast cells to proteasome inhibitors activated Gcn2 which phosphorylated eIF2α and this induced apoptosis (Jiang and Wek, 2005). Another explanation for this finding was the following: Unstructured proteins tend to be degraded more quickly than structured proteins (Babu et al., 2011). The association of Yih1 with the proteasome may thus be due to the presence of the unstructured linker and may reflect a short half-life time of Yih1 before degradation (Wright and Dyson, 1999). Two additional proteins involved in actin function, Sla2 and Vip1, were identified. Another heat shock protein, Hsp78, is present in the mitochondria while Lhs1, a Hsp70 family protein, is found in the endoplasmic reticulum.

[2] Gcn1 was identified with GST-Yih1 in the western blot but not in the mass spectrometry and for this reason was assigned to Set III instead of Set I.

Set IV contains proteins that were found only once in the published data and co-precipitated only with GST-Yih1*2, which could imply a higher likelihood of identifying unspecific interactors compared to the previous sets. This set contains another elongation factor, Tef4 (eEF1Bγ), as well as the mitochondrial maintenance protein Pim1 and both were found in a complex with TAP-Yih1 and GST-Yih1*H2. Additionally, Arp3, Bbc1, Cct8 and Rvs167 are actin-associated proteins while Myo2 is a motor protein involved in actin-based transport of cargos (Pruyne et al., 2004). This set contains several proteins that show an increased abundance under DNA-replication stress, such as Cpr6, Pil1, Rpn2 (coincidentally also part of the proteasome), Stm1 and Zwf1. DNA replication stress can be induced by, for example, urea or oxidative damage to DNA (Burhans and Weinberger, 2007). Interestingly, there is evidence that Gcn2 can act as a protector against both types of stresses (Cai and Brooks, 2011; Chaveroux et al., 2011; Mascarenhas et al., 2008). However, there is no evidence that Gcn2 is activated by DNA damage (Krohn et al., 2008). Cpr6 is related to heat shock proteins as it contributes to Hsp82 activity (Mayr et al., 2000). The inclusion of a number of factors involved in the nuclear import and export of proteins (Crm1, Cse1, Kap95 and Srp1) implies that Yih1 can change its location between nucleus and cytosol. Indeed, there is evidence that Yih1 localises to both cytosol and nucleus (Huh et al., 2003). On one hand this finding may imply that these proteins are involved in a general protein export and that there may not be a role for Gcn2 regulation. On the other hand, Yih1 import and export may be facilitated by other proteins and this could result in a promotion of Gcn2 activity by physically separating the two proteins.

Actin was found in Set V which consists only of proteins that were identified in-house and that were in a complex with both Yih1 types. This set contains a large number of ribosomal proteins, possibly as unspecific interaction but also possibly suggesting the binding location of Yih1 on the ribosome. Hsp104 and additional members of the Hsp70 family were found (Ssa1 and Ssc1) as well as Ydj1 which is involved in the regulation of Hsp70 and Hsp90 function. Kap123 is part of a Kap95-Srp1 nuclear protein import and export complex.

Gcn2 is found in Set VI which consists only of proteins that were found more than once in a complex with Yih1. Only a selection of proteins is shown in Figure C19 due to the high number of proteins identified. Five proteins were found

in a complex with TAP-Yih1 and two co-precipitated Yih1 when they were tagged. Gcn3 is the α subunit of eIF2B, the guanine-nucleotide exchange factor for eIF2 which can be inhibited by eIF2α-P, in turn the target of Gcn2 (Pavitt et al., 1998). Spc72 and Nip100 are components of the cytoskeleton, specifically the microtubules.

Vma8 and Vma13 were identified only as co-precipitators of GST-Yih1*H2 and, just like Vma2 and Vma5, they were identified as intracellular acid regulators. Additionally, *VMA8* and *VMA13* deletion strains were tested positive for SM sensitivity (Parsons et al., 2004). Sac6 is another actin-associated protein.

Aap1, Pop2 and Shp1 were discussed previously because they were most frequently in a complex with Yih1 (cf. Figure C2). However, they were not identified in the in-house interactome. This may suggest that they are false positives. On the other hand this may suggest that their interactions with Yih1 were transient and only occur under certain conditions, for example starvation. It is also possible that the number of interactions that occurred was low, the resulting number of peptides was low and therefore these proteins were not detected as significant by the mass spectrometry. Supporting this argument is the finding that Aap1, Pop2 and Shp1 were only found with a low score by the published study (Krogan et al., 2006).

Notably, Gcn2 and Gcn20 were only identified in the published interactome but not in-house. One reason for this could be because low scoring interactions were included. While it increased the likelihood of identifying specifically interacting proteins these findings suggest that there is still a level of unspecific binding. This would be especially true for proteins that were only found once indirectly with Yih1 and Gcn20 and eIF2α are among them. In the published studies Yih1, Gcn1, Gcn2 and Gcn20 co-precipitated with a tagged Sem1 (a proteasome component). In addition, Yih1, Gcn1 and Gcn2 co-precipitated with Ilv1 and eIF2α. Finally, Yih1 and Gcn1 co-precipitated with Pat1 and She3. Thus, it is possible that Yih1 and a (Gcn20-)Gcn1-Gcn2 complex bound to these tagged proteins independently. It is also possible that their interaction was bridged by other, so far unknown proteins. These may be associated with the ribosome as well but it may involve other cellular components, as the finding of cytoskeleton-associated proteins such as Vps1 or Nip100 suggests. Therefore, a close relationship between these proteins can be detected, even though affinity

purification does not allow the identification of direct interactions. This suggests that the in-house interactome may be more specific because Gcn2 or Gcn20 were not identified. However, Pgk1 was found only with Yih1*H2, supporting the idea that it showed higher affinity for binding other proteins.

Jsn1 was found as a YBP in a yeast two-hybrid by Ito et al. (2001). *jsn1*Δ strains were not tested for a growth defect under starvation conditions so far. There is evidence that it play roles in actin and microtubule organisation (Fehrenbacher et al., 2005; Machesky and Gould, 1999). In addition, overexpression of *JSN1* conferred sensitivity to benomyl, a drug that destabilises microtubules (Machin et al., 1995).

To summarise, approximately one quarter of all proteins that were identified by both the published and in-house studies have a role in protein synthesis. This was expected because of the involvement of Yih1 in translation regulation. Of those, roughly 10 % are ribosomal proteins. In a previous study RPL39 and RPS22 were found to co-precipitate with Yih1 (Waller et al., 2012); however, neither of them was identified in this analysis. In addition, other translational components such as elongation (eEF1A and eEF3) and initiation (eIF3) factors were identified. Another group of functionally related proteins that were identified are proteins involved in amino acid biosynthesis. It was not expected that they are in a complex with Yih1 and the specific involvement of Yih1 in this regulatory pathway is not known. In addition to actin as a known YBP, additional components of the cytoskeleton were identified. This includes tubulin (involved in cell division as it ensures that the chromosomes are separated correctly) and proteins involved in regulating and stabilising the cytoskeleton such as Bbc1 or Sla2 (both actin-binding proteins but only identified with the Yih1 mutant). In addition, the motor protein myosin Myo2 that is involved in actin-based transport of cargos co-precipitated with Yih1*H2.

A number of identified proteins were heat shock proteins and proteins that are associated with the proteasome. These two groups of proteins are usually considered contaminants because they have a wide range of functions and subsequently show a high number of interactions as well as a presence in most organelles (Gavin et al., 2006; Guerrero et al., 2006; Krogan et al., 2006; Trinkle-Mulcahy et al., 2008). Proteins are constantly recycled by the proteasome and heat shock proteins often increase their activity under stress. However, as they

were found in both the published studies as well as in the in-house interactome and considering the known relevance of Hsp90 for Gcn2 function it is possible that these proteins are specific YBP. Therefore, other common contaminants that were identified in the Yih1 interactome were considered as possible YBP. This included proteins such as tubulin, other elongation factors, ribosomal proteins, amino acid biosynthetic proteins (Ura2, Ilv1-6, Met10, Lys12, Ade5,7 etc.) or metabolic proteins (Tdh1 or Gal10).

Interestingly, almost 40% of the proteins that were identified with both approaches localise to the mitochondrion, either exclusively or in addition to other organelles. In comparison, 11% and 25% are localised to the cytosol and nucleus, respectively. For some proteins the exact cellular localisation is unknown or they exist in several compartments. In summary, Yih1 co-precipitated proteins from various cellular compartments and of a diverse range of functions, supporting the idea that Yih1 has more functions than just regulating Gcn2, as suggested previously (Sattlegger et al., 2011). In fact, Yih1 may be able to regulate several Gcn2 functions such as resistance against a number of stresses. One caveat is that during cell lysis the compartmentalisation of the cell is destroyed and proteins that are usually separated from each other come into contact, resulting in false positives (Mackay et al., 2008). Thus, it was possible that a certain fraction of proteins in the Yih1 interactome represent interactions that do not occur in the native physiological environment.

It is not possible to use affinity purification assays to confirm a direct physical interaction between two proteins. This method may also report false positives with higher frequencies compared to other methods such as Y2H, as mentioned above. However, one major goal of this study was to identify proteins that promote Gcn2 function. YBP are used to increase the likelihood of identifying proteins that promote Gcn2 function, possibly through regulation of Yih1 function, regardless of a direct interaction with Yih1 (cf. Figure A8). Therefore, a YBP that was found a false positive but at the same time promotes Gcn2 function and was not characterised as such previously would be an important finding (see following chapters and Chapter A.22).

Almost two-thirds of all proteins that were identified by both the published and in-house studies were smaller than 60 kDa and no protein was smaller than 10 kDa, probably because smaller proteins had fewer peptides available for

identification and thus did not reach the statistical significance threshold for inclusion in the data set. This was observed in the large-scale studies by Krogan et al. (2006) and Gavin et al. (2006) as well. Mass spectrometry analyses were more likely to identify larger proteins (>100 kDa) and less likely to identify smaller proteins, i.e. proteins smaller than 20 kDa. Thus, they concluded that mass spectrometry might be biased because *larger* proteins are overrepresented and *smaller* are underrepresented. Additionally, abundant proteins are more likely to be identified. This was seen in another publication which looked at the yeast proteome (Washburn et al., 2001). This then could suggest that very small and very large as well as less abundant potential YBP have escaped identification.

Chapter D

Finding potential Gcn2 regulators

Chapter D: Finding potential Gcn2 regulators

In the previous chapter putative YBP were identified based on published and in-house protein-protein interaction studies. YBP that promote Gcn2 function should impair the GAAC when they are missing from the cell. This is an indication that they play a role in the cell's starvation response and Gcn2 regulation. In this work, starvation was induced by the drug SM which causes starvation for the amino acids leucine, valine and isoleucine.

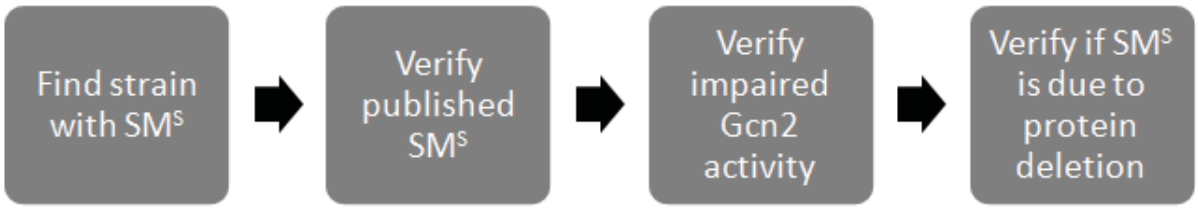


Figure D1: Workflow for this chapter. SM^S: SM sensitivity.

D.1 Published proteins whose deletion results in an impaired starvation response

Identifying deletion strains that showed a growth defect under starvation induced by SM was a first indication that a certain protein is involved in the starvation response. Comprehensive screens that investigated the SM sensitivity of deletion strains were already conducted by others. One study used a library of 651 single-gene deletion strains and scored them for their growth when exposed to a variety of drugs, including SM (Parsons et al., 2004). 218 of the tested proteins were found as published putative and in-house putative YBP (see Appendix B). Of those 218 proteins, 32 were found to be involved in helping the cell overcome starvation stress as shown by growth defects of the respective deletion mutants under SM-induced starvation conditions (Parsons et al. 2004 and Table D1). Another study tested a library of deletion strains for SM sensitivity and reported a selection of their results which consisted of proteins involved in vesicle-mediated intracellular transport (Zhang et al., 2008). Eight of the proteins they tested were found in the Yih1 interactome and all but one deletion strain (*pep1*Δ) were SM sensitive (Table D1).

In total, the deletion of 37 putative Yih1-binding proteins resulted in a growth defect under amino acid starvation conditions that were induced by SM in the published literature (Figure D2, Table D1 and Set VII in Figure D3). This included the known YBP Gcn1 and actin as well as Gcn2 and Gcn20. Only *vma2*Δ and *vma5*Δ strains were found by both Parsons et al. and Zhang et al. as SM sensitive. This low overlap between the two studies was probably due to the low number of proteins investigated. *doa4*Δ, *sac6*Δ, *vps1*Δ and *vps15*Δ strains were tested by both studies but only Zhang et al. found them as SM sensitive. There are several possible explanations for this. For example, evidence is mounting that the strain background is playing a significant role on the outcome of a growth assay and this is something that needs to be taken into account (Liti et al., 2009). We have published supporting evidence for this observation recently (Cambiaghi et al., 2014). In the cited study we tested two different yeast strains, H1511 and BY4741, and while there is some overlap it is clear that each strain is sensitive to different concentrations of a drug or chemical or one of the two is not sensitive at all. However, both Parsons et al. and Zhang et al. used BY4741 strains from the commercial Yeast Knockout Collection. In addition, they used solid SC media to grow cells and therefore it was unlikely that the background strain or the growth media alone can explain the differences. One point of difference was the drug concentration used: Zhang et al. used 0.5 to 1 μg/mL of SM while Parsons et al. used 3 μg/mL. However, despite the lower SM concentration Zhang et al. identified several deletion strains with an impaired growth defect while the same strains were not considered SM sensitive by Parsons et al. (Table D1). Another difference is that Zhang et al. used 10-fold dilutions when spotting cells and Parsons et al. 100-fold dilutions. Parsons et al. repeated the large-scale growth assays three times and manually verified SM sensitive deletion strains with spot assays while Zhang et al. did not provide detailed information on their verification process. Based on the available information it was not possible to conclusively determine the reason for the different results. Therefore, data from both studies were included and for the purposes of this work a deletion mutant was considered SM^s if it was found as such at least once.

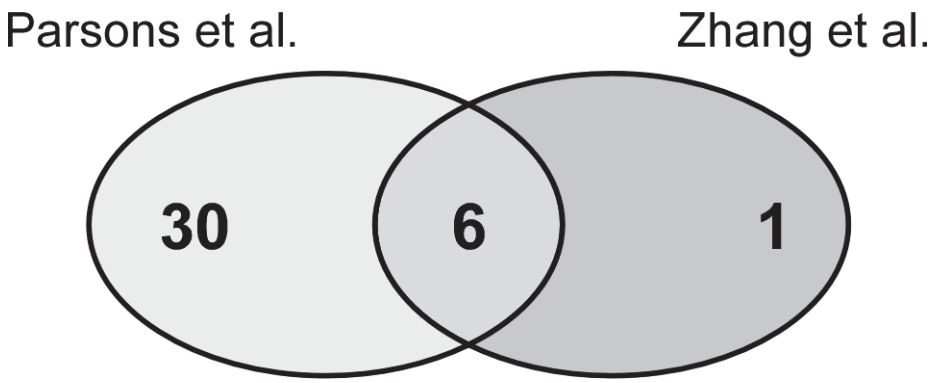


Figure D2: The number of YBP that were tested for and showed SM sensitivity in published studies. Six deletion mutants were tested in both studies and found SM^s in at least one study. See Table D1 for details.

Table D1: 37 published putative YBP whose removal from the cell resulted in an impaired starvation response. An empty cell indicates that the protein was not tested.

Protein	Parsons 2004	Zhang 2008
Bfr1	yes	
Chc1		yes
Cvt9	yes	
Doa4	no	yes
Erg6	yes	
Gal11	yes	
Gcn1	yes	
Gcn2	yes	
Gcn3	yes	
Gcn20	yes	
Grr1	yes	
Ilv6	yes	
Mrpl31	yes	
Nde1	yes	
Nip100	yes	
Pet56	yes	
Rpl14a	yes	
Rpl8b	yes	
Sac6	no	yes

Protein	Parsons 2004	Zhang 2008
Sgf73	yes	
She9	yes	
Sin4	yes	
Snf2	yes	
Snf6	yes	
Snf8	yes	
Spc72	yes	
Spt20	yes	
Top1	yes	
Ure2	yes	
Vma13	yes	
Vma2	yes	yes
Vma5	yes	yes
Vma8	yes	
Vph2	yes	
Vps1	no	yes
Vps15	no	yes
Ypk1	yes	

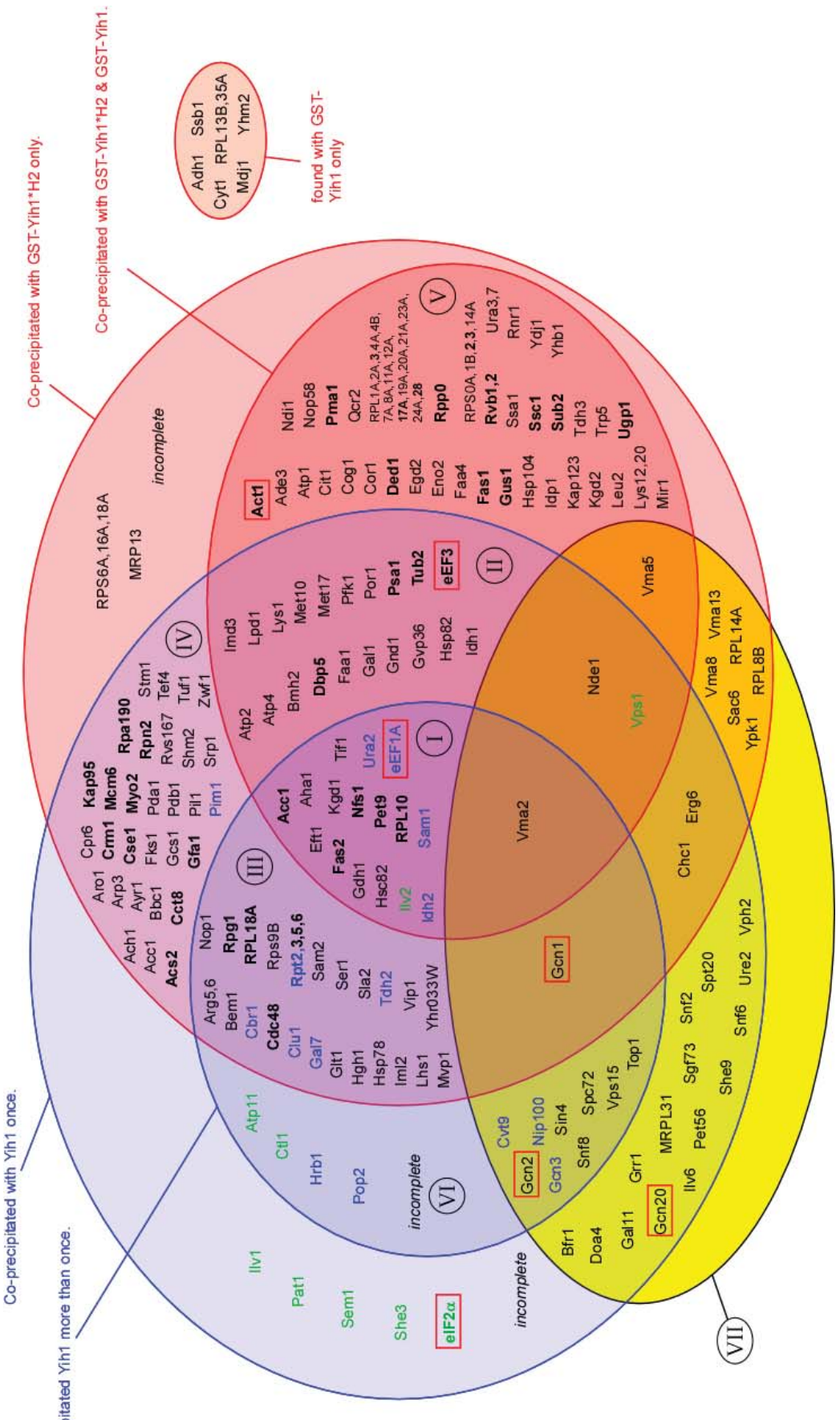


Figure D3: The 37 published putative YBP whose removal from the cell resulted in an impaired starvation response are shown in the yellow circle (set VII).This image is based on Figure C18.

D.2 Verifying the impaired starvation response of 30 deletion strains

The published SM sensitivity of deletion strains was verified in-house. For this, the previously characterised proteins Gcn1, Gcn2, Gcn20 and actin were excluded. In addition, Eft1, Gdh1, Idh2, Kgd1, Tif1 and Ura2 were included because they were found in a complex with Yih1 more than once in the published interactomes and in the in-house interactome were found as co-precipitators with Yih1 and the Yih1 mutant. Some proteins were already studied for their SM sensitivity in independent, parallel projects in our laboratory and were thus excluded as well. The list of the remaining 30 proteins that were tested is given in Table D2. Parts of these experiments were done by Mathias Joachim who was supervised by Michael Bolech.

Table D2: Putative YBP that were used in-house to verify the SM sensitivity. An empty cell indicates that the protein was not tested. The column "Verified" indicates if the deletion strain was SM^S in-house. The column "Set" indicates if a proteins was in a set from Figure C19 or only in published ("pub") or in-house ("in") data.

Protein	Parsons 2004	Zhang 2008	Verified	Set
Bfr1	yes		yes	pub
Chc1		yes	no	IV
Cvt9	yes		yes	VI
Eft1			no	I
Erg6	yes		yes	IV
Gal11	yes		no	pub
Gcn3	yes		yes	VI
Gdh1			no	I
Grr1	yes		no	pub
Idh2			no	I
Kgd1			no	I
Mrpl31	yes		no	pub
Nde1	yes		yes	II
Nip100	yes		yes	VI
Pet56	yes		no	pub

Protein	Parsons 2004	Zhang 2008	Verified	Set
Sac6	no	yes	yes	in
Sgf73	yes		yes	pub
She9	yes		yes	pub
Sin4	yes		yes	pub
Snf8	yes		yes	pub
Spc72	yes		yes	VI
Spt20	yes		yes	pub
Tif1	no		no	I
Ura2			no	I
Ure2	yes		yes	pub
Vma13	yes		yes	in
Vma5	yes	yes	yes	V
Vma8	yes		yes	in
Vph2	yes		yes	in
Ypk1	yes		no	in

The verification was done using a semi-quantitative growth assay (see Chapter B.15). Briefly, yeast strains containing a single gene deletion as well as wild type strains and *gcn2*Δ as controls were grown in appropriate liquid media to saturation. 5 µL of culture was transferred in ten-fold serial dilutions to solid rich media and minimal containing SM and without SM. The plates were incubated at 30˚C for several days and their growth documented regularly using a document scanner. If a protein is involved in the starvation response then cells should be negatively affected under starvation conditions – in other words they should exhibit a growth defect compared to the wild type.

The growth assays are shown in Figure D4 and Figure D5. In order to determine if a deletion strain is SM sensitive the colonies were scored using a ten point system as follows: Two points are given for a fully grown colony and one point for a colony that was visible but has not fully grown. Ten points indicated that colonies were fully grown for all dilutions and zero points indicated that no colonies were visible. The values were normalised to the growth on control (SD media) and the wild type was set to 1.0. This was done for all tested proteins and is summarised in Figure D6.

As expected the wild type strains grew equally well under replete, minimal and starvation conditions because they were able to activate the GAAC and overcame the stress. *gcn2*Δ strains grew on YPD and SD media but showed no growth under starvation conditions (SD+SM), as expected, because cells without Gcn2 are unable to respond and overcome amino acid starvation.

All strains, except for *chc1*Δ, grew similar to the wild type on replete media (YPD). The *chc1*Δ strain showed a strong growth defect under all tested conditions, indicating a slow growing phenotype. For this reason it could not be determined if Chc1 is involved in the starvation response.

Ten strains showed no significant growth defect under starvation conditions compared to the wild type (*eft1*Δ, *gal11*Δ, *ghd1*Δ, *grr1*Δ, *kgd1*Δ, *mrpl31*Δ, *pet56*Δ, *tif1*Δ, *ura2*Δ and *ypk1*Δ), indicating that they were not required for the cell to overcome starvation. Another strain, *gal11*Δ, showed resistance to SM because it grew faster than the wild type under starvation conditions while no growth difference was seen on control plates. This also indicated that the Gal11 protein is not necessary to overcome amino acid starvation.

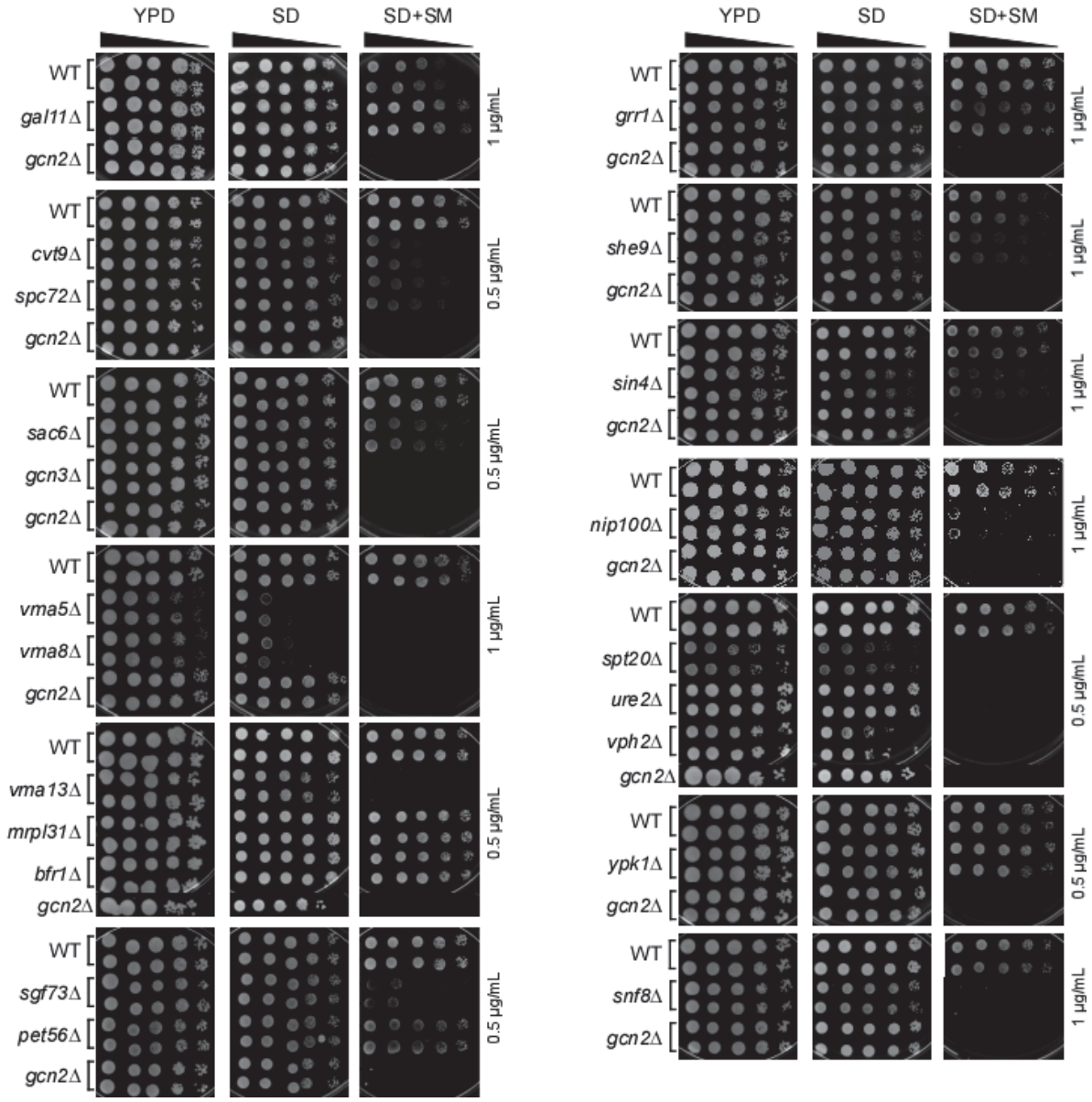


Figure D4: Growth assays, part 1. An impaired starvation response was shown for *cvt9*Δ, *gcn3*Δ, *spc72*Δ, *sac6*Δ, *vma5/8/13*Δ, *snf8*Δ, *she9*Δ, *sin4*Δ, *nip100*Δ, *spt20*Δ, *ure2*Δ, *vph2*Δ and *sgf73*Δ strains. Cells were grown and plated as described in the text. Amino acid starvation was induced by addition of SM at indicated concentrations. Each panel shows the result of a single petri dish.

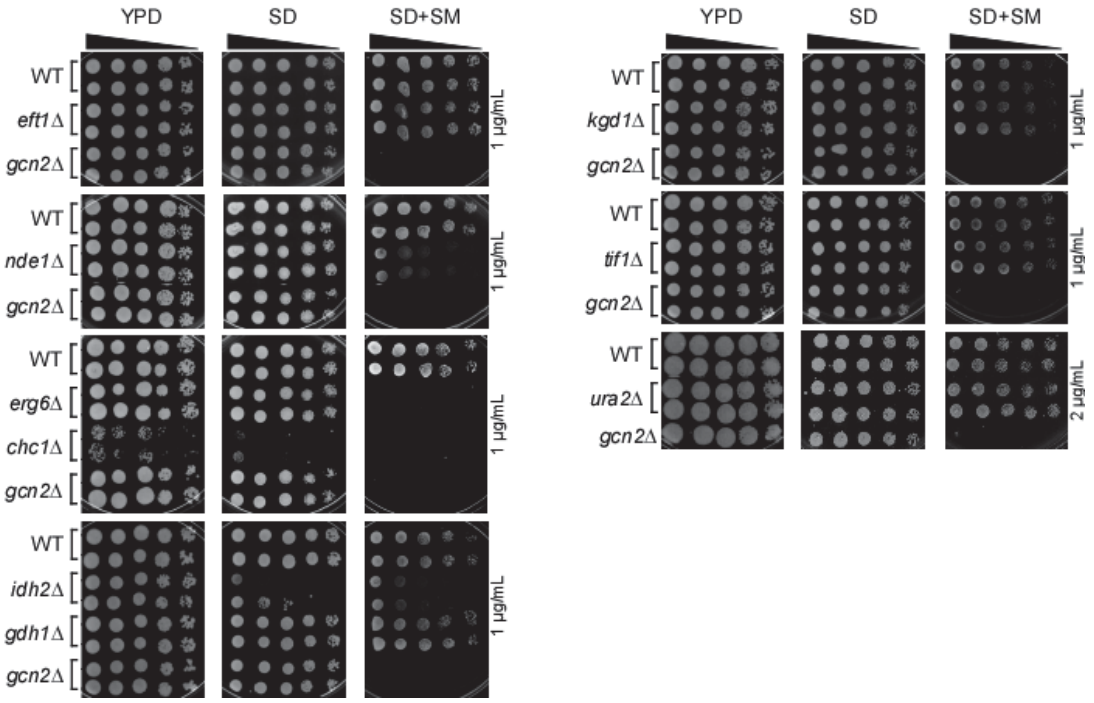


Figure D5: Growth assays, part 2. An impaired starvation response was shown for *nde1*Δ, *erg6*Δ and *idh2*Δ strains. Cells were grown and plated as described in the text. Amino acid starvation was induced by SM concentrations as indicated. Each panel shows the result of a single petri dish.

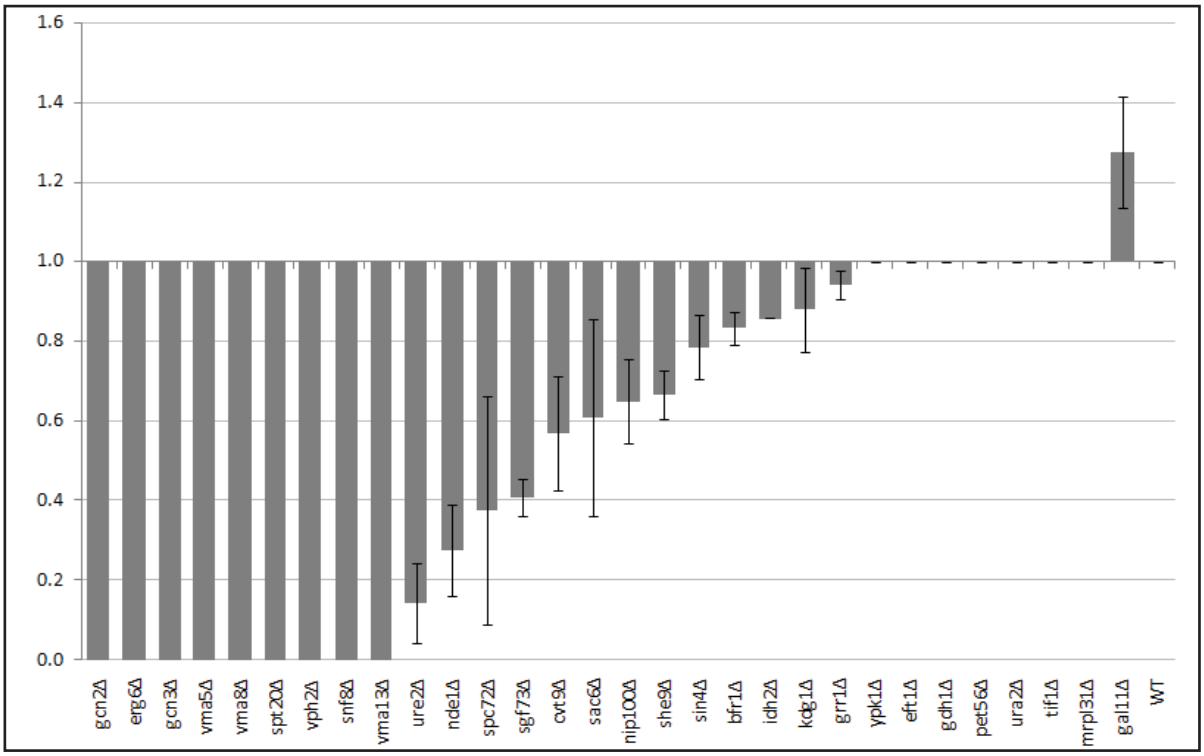


Figure D6: Quantification of the growth assays. Colonies grown under three different SM concentrations were used to determine the growth, as explained in the text. The wild type was set to 1 and all values were normalised accordingly. Values are given as the ratio of minimal media to starvation media. The standard error is indicated. The horizontal axis crosses at 1.0 and values below 1.0 indicate SM sensitivity and values above 1.0 indicate SM resistance. *chc1*Δ strains showed a strong growth defect on minimal and YPD media and were not included here.

Nineteen deletion strains showed a growth defect under starvation conditions. Of those, *idh2*Δ, *spt20*Δ, *vma5*Δ, and *vma8*Δ strains showed a moderate to strong growth defect on minimal media but not on YPD. However, for these strains the growth defect under starvation conditions was stronger than under minimal conditions, indicating that they were indeed SM sensitive (Figure D6).

*cvt9*Δ, *she9*Δ, *sin4*Δ, *spc72*Δ, *vma13*Δ, and *vph2*Δ strains were SM sensitive but also showed a slight growth defect on SD media. Again, the growth defect on SM-containing media was much stronger in comparison, indicating that they are SM sensitive. *bfr1*Δ, *erg6*Δ, *gcn3*Δ, *nde1*Δ, *nip100*Δ, *sac6*Δ, *sgf73*Δ, *snf8*Δ and *ure2*Δ strains showed a growth defect on SM media compared to the wild type and none on control plates, suggesting their involvement in the starvation response.

Most of the verified SM sensitivities were in concordance with the published results. The published impaired growth could not be reproduced for *chc1*Δ, *gal11*Δ, *grr1*Δ, *mrpl31*Δ, *pet56*Δ and *ypk1*Δ. *chc1*Δ exhibited a strong SM sensitivity (Zhang et al., 2008) but also a slow growing phenotype on control plates in this work. This might be due to a difference in media that was used: Instead of minimal media Zhang et la. used SC media which contained all amino acids except Ile and Val. Since more nutrients were available in their case this could support the growth of this slow growing strain. Nonetheless, it was possible that this strain was SM sensitive but this was only seen when the strain was provided with a higher level of nutrition that allowed it to overcome the slow growth on the control media. The other five deletion strains were found as SM sensitive only by Parsons et al. (2004). This study used SC media as well and the same nutritional differences may apply compared to media used in this work. In two cases, *grr1*Δ and *pet56*Δ, they found a weak SM sensitivity but it was not considered as such. There may have been an impaired growth under starvation conditions but it was too subtle and was only detected in their study. On the other hand, *gal11*Δ and *ypk1*Δ strains were moderately SM sensitive in their study but were not found sensitive in this work. A growth defect on minimal and rich control media may have exaggerated the growth defect on starvation media. One study found that *gal11*Δ and *ypk1*Δ strains were growing slow on SC and YPD media (Qian et al., 2012). Therefore, this may be one explanation for the different findings. Interestingly, it

was found that the BY4741 series of yeast strains is sensitive to growth on SC medium but not on SD (Cohen and Engelberg, 2007). This was likely related to the chromosomal *LEU2* deletion in these strains: It was proposed that these cells cannot take up leucine efficiently from the medium because of the overabundance of other amino acids which resulted in a saturation of the transport system (Greasham and Moat, 1973). This was not the case for YPD because the high amino acid concentration likely was balanced by the high level of other nutrients that were present (Cohen and Engelberg, 2007). Supporting this, supplementing SC medium with yeast extract restored growth of BY4741 strains but not when supplemented with peptone (Cohen and Engelberg, 2007). Therefore, depending on the intensity of the growth defect this may have masked an SM sensitivity or exaggerated it. This may help explain another discrepancy: *sac6*Δ strains was shown to be SM sensitive in this work and by Zhang et al. but not by Parsons et al. Strains deleted for *SAC6* showed a growth defect in SC medium and presumably it was considered SM sensitive by Zhang et al. but not by Parsons et al.

To conclude, 19 out of the 30 tested deletion strains were verified for their SM sensitivity. This suggested that the starvation response in these strains was impaired which suggested that Gcn2 function was reduced.

D.3 Spc72, Idh2, Vma5 and Vma8 are necessary for Gcn2 activation under starvation conditions

An impaired growth under amino acid starvation conditions could indicate the inability of a deletion strain to activate Gcn2. However, the growth defect could also be caused by events independent of Gcn2 because the deleted protein is involved in other important cellular functions or the deleted protein regulates the starvation response downstream of Gcn2. In order to test this the level of Gcn2 activity was measured. If Gcn2 is active then it phosphorylates eIF2α (eIF2α-P). If the eIF2α-P level in a certain deletion strain is lower than in the wild type then this suggest that Gcn2 activation is reduced. This would strongly suggest that the deleted protein is required for promoting Gcn2 activation. The 19 deletion strains that showed SM sensitivity were used. Parts of these experiments were done by Mathias Joachim who was supervised by Michael Bolech.

Cells were grown in minimal media until they reach OD_{600} = 0.6. Cells were starved for 1 h by adding SM and this was followed by adding formaldehyde (1% final concentration) to crosslink proteins for another hour. The crosslinking was stopped by adding glycine. Subsequently cells were broken and 10 µg of protein was separated via SDS-PAGE. The proteins were transferred onto PVDF membranes and exposed to antibodies against eIF2α-P. Antibodies against Pgk1 were used to test for equal loading. Two independent colonies of each deletion strain were used and the wild type was used in triplicate. *gcn2*Δ strains were used as a negative control. These experiments were done for all nineteen deletion strains that showed SM sensitivity and for *gal11*Δ strains which showed SM resistance, shown in Figure D7. The eIF2α-P and Pgk1 signals for each strain were quantified using the ImageJ software (Schneider et al., 2012) and the eIF2α-P/Pgk1 ratio was calculated (Figure D8). Student's t-test was done and a value below 0.05 was considered statistically significant.

For all experiments the starved wild type showed an increased eIF2α-P signal as compared to unstarved conditions, as expected, because Gcn2 was activated in this strain (Figure D7). As expected, *gcn2*Δ strains showed no or a weak basal signal for eIF2α-P (Dever et al., 1992).

The eIF2α-P signal for *spc72*Δ, *idh*Δ*2*, *vma5*Δ and *vma8*Δ strains was significantly reduced under starvation conditions compared to the wild type (Figure D8). This indicated that Gcn2 activity was reduced in these strains and subsequently eIF2α phosphorylation was reduced. This then suggested that these proteins are involved in promoting Gcn2 activation.

*vph2*Δ, *sgf73*Δ and *gal11*Δ strains showed a small reduction in eIF2α-P levels but it was not significant. Similarly, *ure2*Δ, *sin4*Δ, *she9*Δ and *cvt9*Δ strains showed a small but insignificant increase in eIF2α-P levels. *nip100*Δ, *nde1*Δ and *erg6*Δ strains did not show a change in eIF2α-P levels. This suggested that these proteins are not involved in promoting Gcn2 activity.

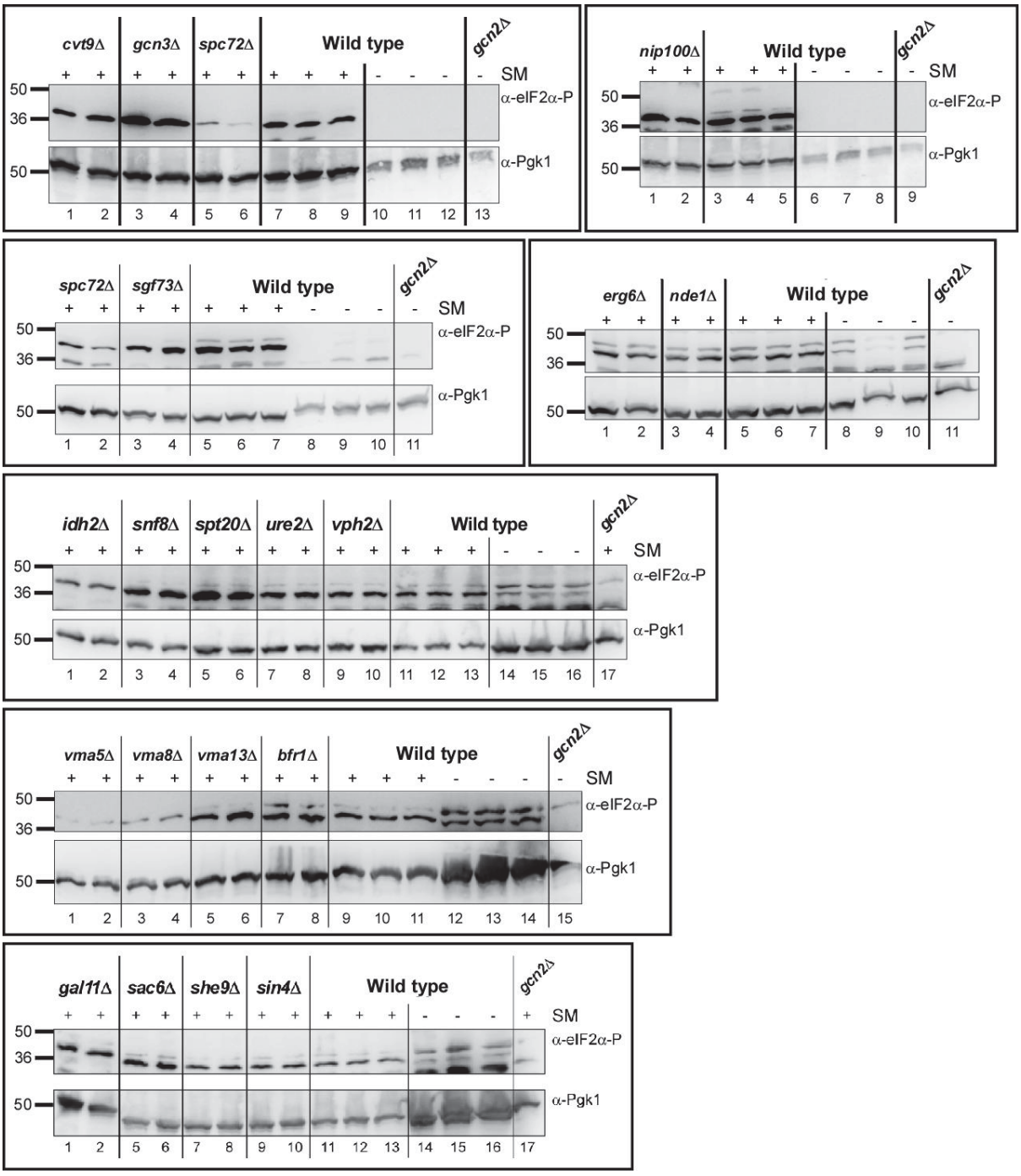


Figure D7: *spc72*Δ, *idh2*Δ, *vma5*Δ and *vma8*Δ strains show reduced eIF2a-P levels under starvation conditions compared to the starved wild type, indicative of reduced Gcn2 activity. Deletion strains and the wild type were grown in minimal media to saturation, starved with 2 µg/mL SM for 1 h, harvested and proteins were separated via SDS-PAGE. Western blots were done using antibodies against eIF2α-P and Pgk1.

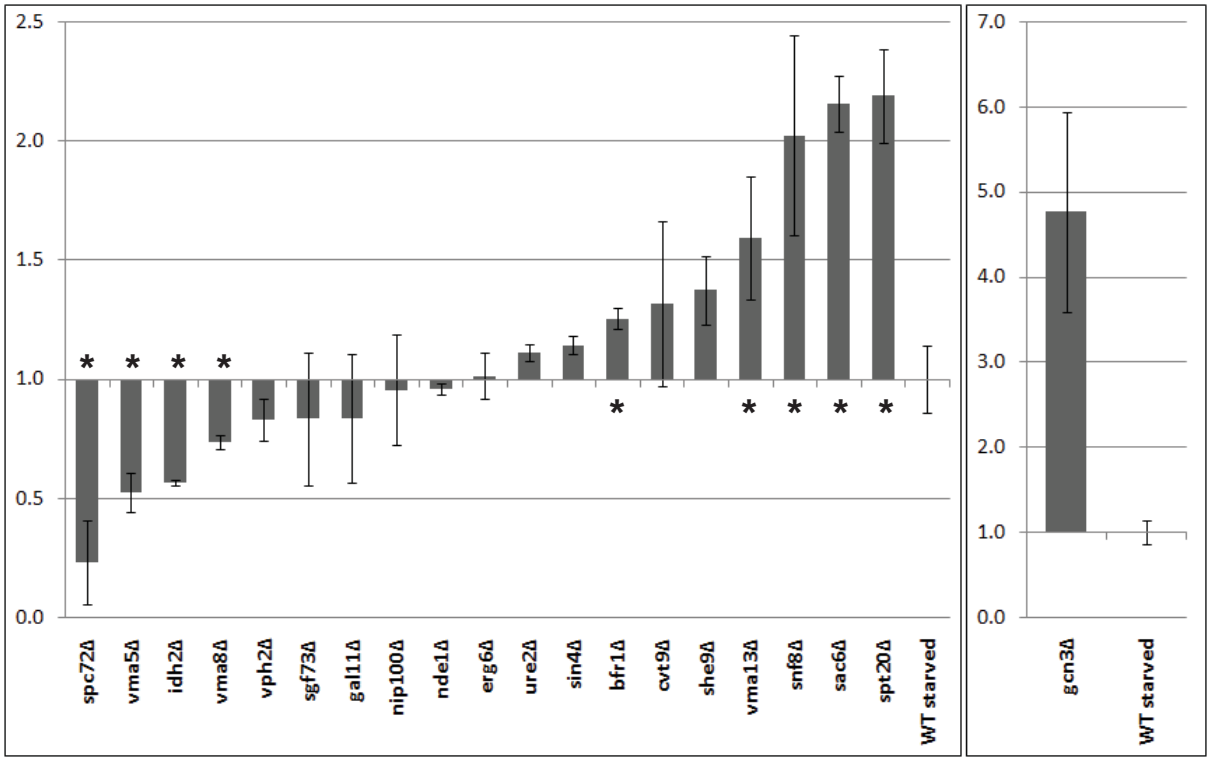


Figure D8: *spc72*Δ, *idh*Δ*2*, *vma5*Δ and *vma8*Δ strains show reduced eIF2α-P levels under starvation conditions compared to the starved wild type, suggesting reduced Gcn2 activity. The western blot signals for all tested strains were quantified from two independent cultures using ImageJ image software and the ratio of eIF2α-P to Pgk1 was calculated. The wild type was set to 1 and all values are normalised accordingly. The standard error is given. The horizontal axis crosses at 1.0 and values below 1.0 indicate reduced eIF2α-P levels and values above 1.0 indicate increased eIF2α-P levels compared to the starved wild type. An asterisk indicates a significant difference to the wild type ($p = <0.05$). *gcn3*Δ is shown in a separate graph on the right due to its relatively high values and standard error.

Almost half of the proteins tested showed an increased eIF2α-P level (Figure D8), suggesting an increase in Gcn2 activity. This was unexpected because these strains were SM sensitive which suggested a reduced Gcn2 activity and therefore reduced eIF2α-P levels. One explanation could be that the SM sensitivity that was seen in the growth assays was not caused by an impaired Gcn2 function. Instead, the respective protein is involved in other cellular functions that are needed in order to survive the starvation. Another explanation was that the GAAC is impaired downstream of Gcn4. Thus, Gcn2 may be hyperactive in order to compensate for it, increasing eIF2α phosphorylation. Consequently, the strain was unable to respond to the stress and grew slowly.

The protein phosphatase Glc7 reduces eIF2α phosphorylation and acts in opposition to Gcn2, as previously mentioned in Chapter C.1 (Wek et al., 1992). It is possible that a YBP may act as a regulator (either positive or negative) of Glc7.

The removal of this protein would therefore affect Glc7 activity and, in turn, change eIF2α phosphorylation levels independent of Gcn2 activity. Therefore, a YBP would not play a role in the starvation response despite a reduction in eIF2α phosphorylation levels for the respective deletion strain. One way to address this would be to measure Gcn2 autophosphorylation levels because they positively correlate with eIF2α phosphorylation levels (Romano et al., 1998). In addition, Gcn2 is not a known target of Glc7. Therefore, if both Gcn2 and eIF2α phosphorylation levels are reduced in a deletion mutant then this was likely due to the deletion of the protein and not due to Glc7 phosphatase activity.

To conclude, *spc72*Δ, *idh*Δ*2*, *vma5*Δ and *vma8*Δ strains showed reduced eIF2α-P levels under starvation conditions, suggesting a reduced Gcn2 activity. This suggested that they are involved in promoting Gcn2 activity and that this may be mediated by Yih1. Because Yih1 is a Gcn2 inhibitor they would need to inhibit Yih1 to allow Gcn2 activation. Conversely, in the deletion strains Yih1 was not inhibited anymore and was able to inhibit Gcn2. Of those four proteins Idh2 was the only one that was identified by both published and in-house studies (cf. Figure C19). Spc72 was identified as co-precipitator of Yih1 by Krogan et al. (2006) more than once. Vma5 co-precipitated with both GST-Yih1 and GST-Yih1*H2 while Vma8 co-precipitated only with GST-Yih1*H2 in the in-house interactome. These results indicated that both the published and in-house interactome allowed the identification of potential YBP and of proteins that are possibly involved in promoting Gcn2 activity. Furthermore, all four proteins were found as low scoring interaction with Yih1 by Krogan et al. (2006), providing evidence that the approach to include non-significant interactors was suitable for this work. In the growth assays in this work the focus was on proteins that were found more than once in the published interaction and that were found both with Yih1 and Yih1*H2 as well as on a limited number of deletion strains that were found as SM sensitive by previous studies. Thus, it was expected that additional deletion strains for proteins found in the Yih1 interactome show a reduced Gcn2 activity, especially considering not all deletion strains were tested for SM sensitivity in the published literature. In addition, the contradictory results for some deletion strains in the studies by Parsons et al. and Zhang et al. suggested the importance of verifying their SM sensitivity (or lack of).

D.4 Spc72 is involved in helping the cell overcome amino acid starvation

D.4.1 The growth defect of *sp72*Δ strains under starvation conditions can be restored by adding an *SPC72*-containing plasmid

As seen in the previous section deletion of *SPC72* resulted in an impaired growth on SM-containing media and reduced Gcn2 activity. In fact, the level of reduction was the strongest of all the proteins that were tested. Therefore, this protein is the strongest candidate for Yih1-binding and/or Gcn2 activation. However, deletion of *SPC72* could lead to secondary effects that would result in a slow-growing strain under starvation conditions. The deletion strains used in this work were from the widely used yeast knockout collection and one recent study tested them for the presence of secondary mutations. It was found that deleting a gene can cause mutations in one or two other genes and this affected their ability to grow under stress conditions such as low amino acid concentration or heat stress (Teng et al., 2013). Therefore, the next question was whether the growth defect was indeed due to the deletion of *SPC72*. If this is the case then reintroducing the gene into the cell should rescue the growth defect and the cells should grow as the wild type under starved conditions. Conversely, if the growth defect remains then it was likely due to secondary mutations or other secondary effects. For this complementation assay, a plasmid containing *SPC72* was transformed into *spc72*Δ strains. This plasmid was part of the Yeast Genomic Tiling Collection which contains 10 kb segments of the yeast genome (Figure D9) (Jones et al., 2008). This introduced additional gene copies in addition to *SPC72* and could affect the cell in unexpected ways. One way to test for such effects was to introduce the same plasmid into the wild type. If the plasmid has negative effects on the cell that, for example, result in a growth defect then the growth defect should be seen for the wild type as well. For a true complementation test only the respective gene must be reintroduced into the cells but use of the Genomic Tiling Collection allowed a quick initial test whether a genomic fragment containing the gene deleted in the strain can rescue the growth defect caused by SM exposure.

After introducing the plasmid or the empty vector as control into the *spc72*Δ strains and the wild type the SM sensitivity growth assay was repeated as shown in Figure D10. *spc72*Δ strains plus vector showed a strong growth defect on SM-containing plates, as shown previously. This indicated that the vector has not affected growth. Reintroducing *SPC72* on a plasmid abolished this growth defect and the cells grew as well as the wild type under starvation conditions. Furthermore, the wild type that was transformed with the same plasmid was not affected in its growth, indicating that the plasmid did not have a negative effect on the cells under starvation or non-starvation conditions. This was in agreement with the idea that the impaired starvation response of *SPC72* deletion strains was due to removal of this protein.

It was possible that the restoration of the growth defect was due to the other genes on the plasmid, *OAF1*, *YAL049C*, *GEM1*, *YAL047W-A*, *YAL046C*, *YAL045C* and *YAL044W-A*. No obvious relevance to the GAAC is known for the proteins Oaf1, YAL049C and Gem1 and no growth defect or increased growth due to their overexpression was reported previously. In addition, the functions of the latter four ORFs are unknown. This suggested that *SPC72* complemented the growth defect of *spc72*Δ strains. That said, in order to confirm that complementation is indeed due to Spc72 the next step would involve a plasmid that only contains *SPC72* and no other genes. However, time constraints did not allow construction of this plasmid and a previously published plasmid was not available (Knop and Schiebel, 1998).

Figure D9: Gene map of the plasmid containing *SPC72*. Gene names in square brackets indicate a partial gene.

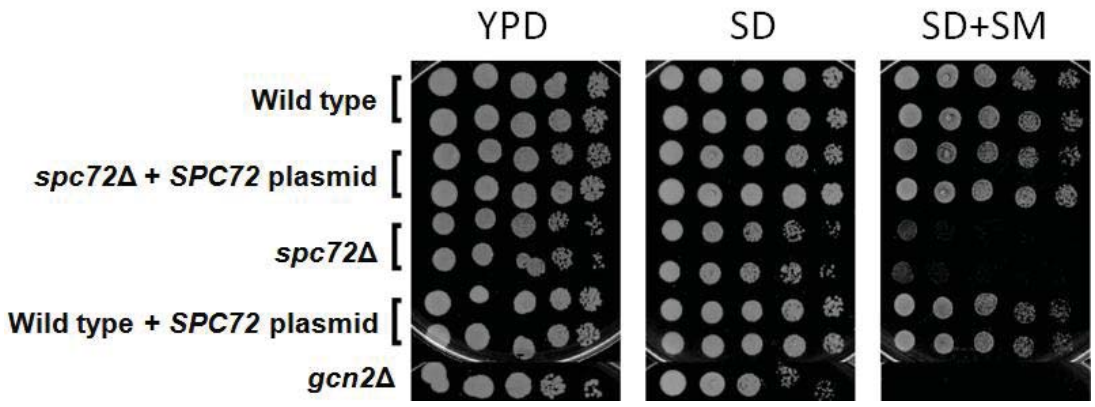


Figure D10: Reintroducing Spc72 into spc72Δ strains restores their growth to wild type levels. This suggests that the growth defect of *spc72*Δ seen under starvation conditions is due to *SPC72* deletion. *SPC72* was introduced into *spc72*Δ strains and the wild type on a plasmid which contains a number of other genes (see text for details). Strains without the plasmid contain an empty vector. Each panel shows the result of a single petri dish.

D.4.2 What effect has deletion of *YIH1* in *SPC72* deletion strains?

Evidence suggested the involvement of Spc72 in the starvation response and Gcn2 regulation. It was investigated if this function of Spc72 is mediated by Yih1. If this is the case then deleting *YIH1* in these strains should restore the growth defect and restore the decreased Gcn2 activity to wild type levels under starvation conditions. For this a strain was used that is deleted for both Spc72 and Yih1 proteins and its growth and Gcn2 activity was compared to the individual single deletions under starvation conditions.

An attempt was made to replace the *YIH1* gene in a *spc72*Δ strain by using a *yih1*::*hisG-URA3-hisG* disruption cassette (Sattlegger et al., 2004). However, this was unsuccessful. Therefore, a different approach that involved mating of yeast was used.

Budding yeast has two mating types, MATa and MATα. When cells of opposing mating type come together they spontaneously fuse and form diploid cells (Merlini et al., 2013). Under low nutrient conditions these cells undergo sporulation and form tetrads, each containing four haploid spores. The *SPC72* MATa (Giaever et al., 2002) and the *YIH1* MATα (RRY42) deletion strains were mated (cf. Chapter B.15 and Table B3). These two strains were auxotroph for methionine and lysine, respectively. In addition, in *yih1*Δ strains the *YIH1* gene was replaced by the *URA3* marker and in *spc72*Δ the *SPC72* gene was replaced by *KanMX4* which confers resistance against the drug G418 (Giaever et al., 2002).

This allowed diploids to grow on minimal medium lacking uracil, methionine and lysine as well as on replete medium containing G418 (Figure D11, “Mating”). To remove any residual haploids, cells were picked and streaked for single cell colonies on selection media and then transferred to replete media. Cells were picked from these plates and transferred to sporulation media to induce meiosis and sporulation (“Sporulation”). The resulting tetrads were then mechanically separated using a micromanipulator device (“Tetrad dissection”). Tetrads were transferred to medium lacking uracil or containing G418 to select for the double deletion. Due to the segregation of the chromosomes and the individual auxotrophies and resistances of the spores it was expected that two of the four spores grow while two should not grow (2:2 segregation). However, the selection for diploids that are deleted for *YIH1* and *SPC72* at the same time was unsuccessful. This tetrad dissection was repeated for sixteen independent spores but all tetrads grew equally well under all conditions. Only for one set of tetrads a selective growth was seen. From this set one tetrad grew on both minimal selection media and media containing G418, suggesting a possible *spc72*Δ/*yih1*Δ double deletion (“Selection”). However, no further work was done using these cells due to time constraints.

Spc72 is not essential but, even though haploids can mate and form diploids, chromosome segregation is abnormal in *spc72*Δ strains (Souès and Adams, 1998). It is possible that diploid formation is still possible but this resulted in subsequent growth defects. Previous studies attempted to delete Spc72 using mating and sporulation. They found that spores deleted for *SPC72* grew slow (Chen et al., 1998; Souès and Adams, 1998). In addition, certain heterozygous strains deleted for one copy of *SPC72* and one copy of another gene did not grow at all (Hoepfner et al., 2002). However, these studies found a 2:2 segregation and thus cannot explain the results seen in this work. Alternatively, growth under all the tested conditions could indicate that the majority of cells underwent mitosis instead of meiosis. Thus, four diploid cells that stuck together may have been dissected by chance instead of tetrads.

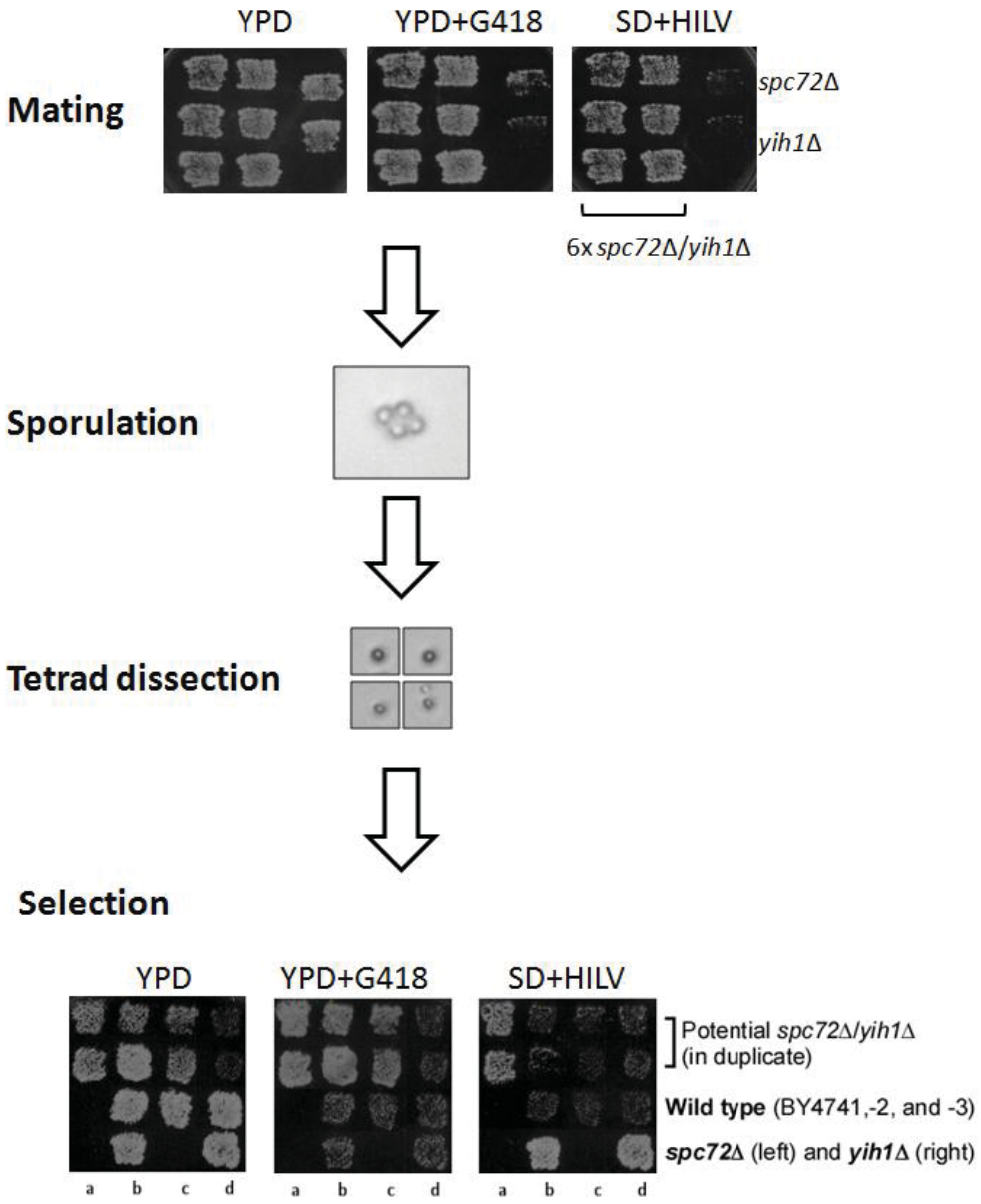


Figure D11: Overview of the workflow to make *spc72*Δ/*yih1*Δ double mutants. *spc72*Δ and *yih1*Δ were successfully mated as confirmed by their growth on selection media while the haploid cells are unable to grow (six different colonies are shown). The diploids were allowed to sporulate and the tetrads were dissected (shown are visible-light microscopic images). The spores of each tetrad (a to d) were transferred to selection media to test for double deletions. The majority of cells grew equally well under all conditions and there was no selection, except for the tetrads shown here. The cells under column a may be *spc72*Δ/*yih1*Δ double deletions.

D.4.3 Spc72 is a spindle pole body component with important roles in microtubule organisation

Spc72 is involved in the development of microtubules which are necessary for proper chromosome segregation, cell polarisation, cellular movement or organelle positioning. Specifically, Spc72 is part of the spindle pole body, the yeast microtubule-organizing centre that is located in the nuclear membrane and that connects the cytoplasmic and nuclear (chromosomal) microtubules (Wigge et al., 1998). The spindle pole body is the functional equivalent of the animal centrosome although there are important structural differences. Spc72 is part of the outer plaque of the spindle pole body and acts as a docking station for the γ-tubulin complex (Tub4, Spc97 and Spc98) (Knop and Schiebel, 1998). Spc72 is essential for karyogamy in mating cells but plays only minor roles during vegetative growth (Pereira et al., 1999).

Spc72 was found to bind Kar1, a protein that recruits Spc72 to the spindle pole body in response to a mating factor (Pereira et al., 1999). In addition, Spc72 was found as a binding partner of Kar3, another component of the spindle pole body and with essential functions during mating (Gibeaux et al., 2013). Thus, it would be expected that they are found in the Yih1 interactome. However, only Kar3 was found once in a complex with TAP-Sem1 and Yih1 by Krogan et al. 2006. The Spc72-Kar1 interaction occurs during G_1 phase of the cell cycle and karyogamy (Pereira et al., 1999). Therefore, it is possible that the Spc72-Kar1 or Spc72-Kar3 interaction did not occur under the conditions that were used by Krogan et al. or by this work. During S/G_2 phase of the cell cycle Spc72 instead binds to Nud1 (Grueneberg et al., 2000) but his protein was also not identified in the Yih1 interactome. As Kar1, Kar3 and Nud1 are part of the microtubule organising centre it is possible that a complex containing these proteins was too large to purify. This could also help explain why Spc72 was only identified in a complex with Yih1 twice in the published large-scale studies but not in the in-house interactome.

As proper microtubule formation is essential for cell viability it was expected that deletion of *SPC72* would be lethal. However, that is not the case. While *SPC72* deletion results in abnormal spindle positioning, aberrant nuclear migration and defective mating capability these cells are able to proliferate, albeit at a slower

pace (Hoepfner et al., 2002; Souès and Adams, 1998). The slow growth was, to a certain extent, reproduced in this work. As Spc72 is part of the microtubule-organising complex and as it was found in a complex with Yih1 this suggested that Gcn2 regulation by the cytoskeleton could be mediated via Yih1.

Interestingly, Spc72 showed yeast two-hybrid interactions with Nip100 and Nde1, two proteins that are associated with microtubules (Wang et al., 2012). In addition, *nip100*Δ and *nde1*Δ strains were SM sensitive as shown by Parsons et al. (2004) and this was verified in this work. However, neither of them showed reduced Gcn2 activity levels. This suggested that they are not involved in promoting Gcn2 activity.

D.4.4 TACC is the mammalian homolog of Spc72

In order to investigate the evolutionary conservation of Yih1 and YBP a first step is to find to homologous proteins in mammals, in this case for Spc72.

The Aurora proteins are a family of mitotic serine/threonine kinases that are conserved from yeast to mammals and are involved in proper chromosome segregation and completion of cell division (Ruchaud et al., 2007). In yeast they establish a connection between the spindle pole body/microtubules complex and kinetochores during cell division (Tanaka, 2005). One study attempted to construct a protein-protein interaction network for Aurora proteins in humans (Tien et al., 2004). This was based on Aurora protein interaction data from yeast and made use of available data on protein sequence homology as well as similar localisation and function. They identified the TACC (Transforming Acidic Coiled-Coil) proteins TACC1, TACC2 and TACC3 in humans as functional homologs of Spc72. Both Spc72 and TACC need to bind to Stu2 or its homolog CH-TOG in humans, both are acidic and both contain a conserved coiled-coil domain, a dynamic protein-protein interaction site (Strauss and Keller, 2008). In addition, they found a significant level of sequence homology between the two proteins when comparing yeast and humans and in other model organisms. So far, other TACC homologs have been found in a number of eukaryotes, including *Schizosaccharomyces pombe*, *Drosophila melanogaster, Caenorhabditis elegans*, and *Xenopus laevis* (Ha et al., 2013). While the functions of the TACC proteins have not been fully

established yet they are known to play roles in microtubule organisation, just like Spc72 (Gergely et al., 2000a, 2000b).

A first step to study the evolutionary conversation of Spc72 function would be to introduce one of the TACC proteins into yeast strains deleted for *SPC72*. As there are structural differences between the microtubule organisation centres in yeast and mammals Spc72 and TACC have relatively low, but nonetheless significant, sequence similarity. Despite this, if TACC is indeed the functional homolog it should be able to perform the same function in yeast. If that is true then expression of TACC at Spc72 levels in a *SPC72* deletion strain should not result in an impaired growth or reduced Gcn2 activity under starvation conditions. In addition, overexpression of TACC in a *SPC72* deletion strain should result in a growth defect, as was found for overexpression of *SPC72*. Furthermore, TACC should be able to interact with Yih1 as well as with the mammalian homolog IMPACT.

While Spc72 was identified in the Yih1 interactome and is likely involved in the starvation response it still needs to be determined if this is mediated via Yih1. A first step to study this is to investigate if there is a direct physical interaction between Spc72 and Yih1. If so then purified Spc72 should co-precipitate purified Yih1 and *vice versa*, in a similar fashion as was done for the Yih1-Gcn1 interaction (Sattlegger et al., 2011). If there is an interaction between Yih1 and Spc72 then the Yih1 fragments can be used in order to investigate the region of Spc72 binding. Another approach is as follows: Four conserved residues were found in the ancient domain of Yih1, suggesting a conserved protein binding surface (Sattlegger et al., 2011). It is possible that Spc72 binds Yih1 in this region. If so, then mutation in one or more of the conserved amino acids should reduce their interaction. Furthermore, it would be interesting to test how this affects the starvation response and the level of Gcn2 activity.

Another way to further study the role of Spc72 in the cellular starvation response is to overexpress it. A strong overexpression of *SPC72* from a 2 µm multicopy plasmid was lethal although a moderate overexpression allowed cells to grow at a reduced rate (Knop and Schiebel 1998). Interestingly, concurrent overexpression of *TUB4*, *SPC97* and *SPC98* restored the growth defect of *SPC72* overexpression to some extent. In addition, overexpression resulted in the formation of large polymers at the spindle pole body. For these reasons it might be

difficult to study SPC72 overexpression unless its levels are kept moderate or TUB4, SPC97 and SPC98 are overexpressed at the same time. Another option is to overexpress Yih1 instead in spc72Δ strains. Overexpression of Yih1 is needed in this case because cells are able to activate Gcn2 when Yih1 is at native levels (Sattlegger et al. 2004). While a high level of Yih1 already confers SM sensitivity the deletion of SPC72 might increase this growth defect.

D.5 Idh2 may be involved in the starvation response

The deletion of *IDH2* resulted in the third-strongest reduction in Gcn2 activity, after *SPC72* and *VMA5* deletions (Figure D8), and a subtle but significant SM sensitivity (Figure D6). As for Spc72, it was tested if this effect was due to the deletion of *IDH2*. Two plasmids from the Tiling Collection that contain *IDH2* were used. While these two plasmids contain different yeast fragments some genes are found on both (Figure D12). Thus, if the growth defect of *idh2*Δ strains is restored with both plasmids then this increases the likelihood for complementation by plasmid-borne *IDH2*.

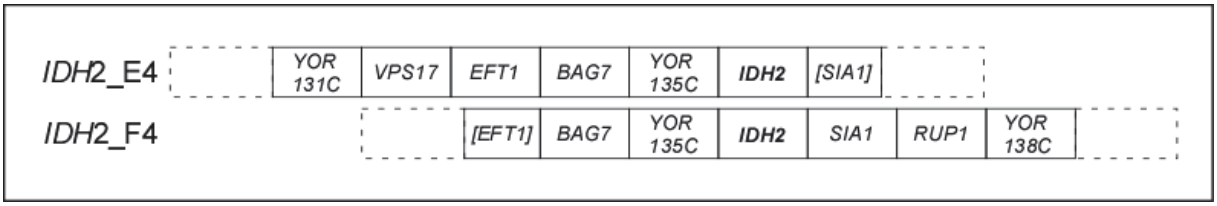


Figure D12: Gene map of plasmids *IDH2*_E4 and *IDH2*_F4. Both plasmids contain *IDH2, BAG7* and *YOR135C*. Gene names in square brackets indicate a partial gene.

D.5.1 The weak growth defect for *idh2*Δ strains under starvation conditions can be restored by adding an *IDH2*-containing plasmid

The result of the growth assay is shown in Figure D13. The colony growth was scored as before and is shown in Figure D14. *idh2*Δ strains containing the vector control showed a slight growth defect on starvation media compared to the wild type and compared to *idh2*Δ strains on the control media, as was seen in the previous growth assays. *idh2*Δ strains that were transformed with either of the two *IDH2*-containing plasmids ("*IDH2*_E4" and "*IDH2*_F4" in Figure D13) showed a slight growth defect on SD control plates compared to *idh2*Δ strains that were

transformed with an empty vector as control. Under starvation conditions, however, the *idh2*Δ strains with the *IDH2*-containing plasmids showed a similar growth defect to *idh2*Δ strains with the vector. Taking into account their growth on minimal media this suggested that the growth defect under starvation conditions in strains deleted for *IDH2* was complemented to some extent by the *IDH2*-containing plasmid (Figure D14). As the level of complementation was similar for both plasmids this indicated that the complementation was a result of the expression of genes that were present on both plasmids. As *IDH2* is found on both plasmids this result suggested that it complemented the growth defect of *idh2*Δ strains.

The wild type that was transformed with either of the two plasmids showed a growth defect on both control and SM-containing media compared to the wild type with the vector (Figure D13 and Figure D14). This may be caused by inhibitory effects of the genes that were expressed from the plasmid. This can be accounted for by determining the growth ratio of control to starvation condition. The growth defect for the wild type containing either of the two *IDH2* plasmids on control media was similar to *idh2*Δ strains. But under starvation conditions the growth defect was slightly stronger for the wild type than for *idh2*Δ strains. In other words, transformation of the wild type with either of the two plasmids resulted in a growth defect compared to the wild type containing the vector (Figure D14). Importantly, transforming *idh2*Δ strains with either of the two plasmids resulted in an *increase* in growth compared to *idh2*Δ strains containing the vector (Figure D14). Therefore, this further suggested that *idh2*Δ strains were complemented by *IDH2*.

As mentioned, it was possible that the slow growth for strains that were transformed with the plasmids was caused by one of the genes that were common to both plasmids. As the Tiling Collection plasmids are expressed at high levels this might be one explanation for the slow growth of the transformed strains. *IDH2* at overexpressed levels had the same activity as natively expressed *IDH2* and thus it is unlikely that this was the cause of the slow growth (Cupp and McAlister-Henn, 1991). The other two genes that were common to two both plasmids were *BAG7* and *YOR135C* (also called *IRC14*). Overexpression of *YOR135C* did not affect cell growth but overexpression of *BAG7* resulted in a slow growth phenotype (Yoshikawa et al., 2011). Therefore, it is likely that the high expression level of

BAG7 may have restricted growth of the transformed strains. In addition, this might mask the restorative effect of reintroducing *IDH2* to some extent, suggesting that the growth of the transformed *idh2*Δ strains might be restored further without simultaneous *BAG7* overexpression.

To conclude, *idh2*Δ strains showed a slight growth defect under starvation conditions and this was likely complemented by reintroducing *IDH2*.

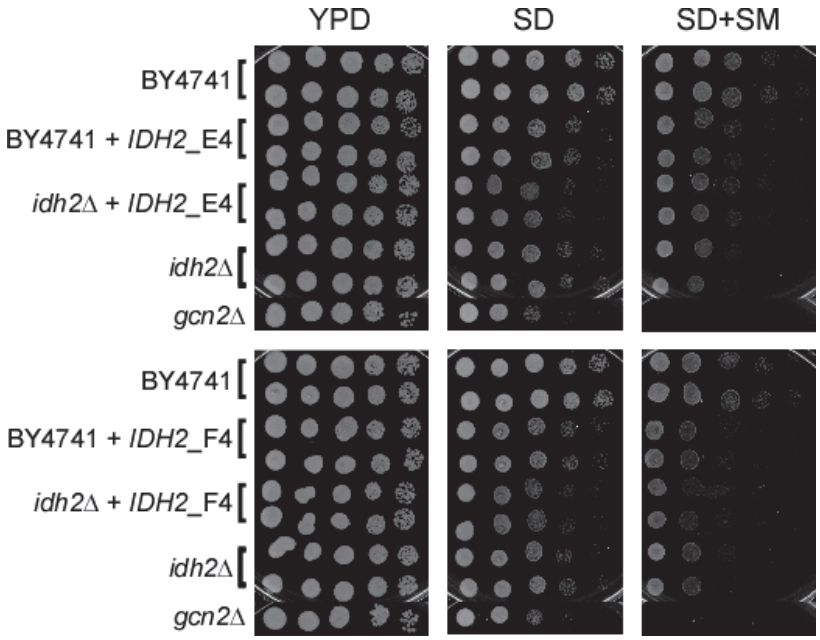


Figure D13: The growth defect of *idh2*Δ strains was restored with a plasmid containing *IDH2* (*IDH2*_E4 and *IDH2*_F4). BY4741 is the wild type strain. Stains without the plasmid contain an empty vector. Each panel shows the result of a single petri dish.

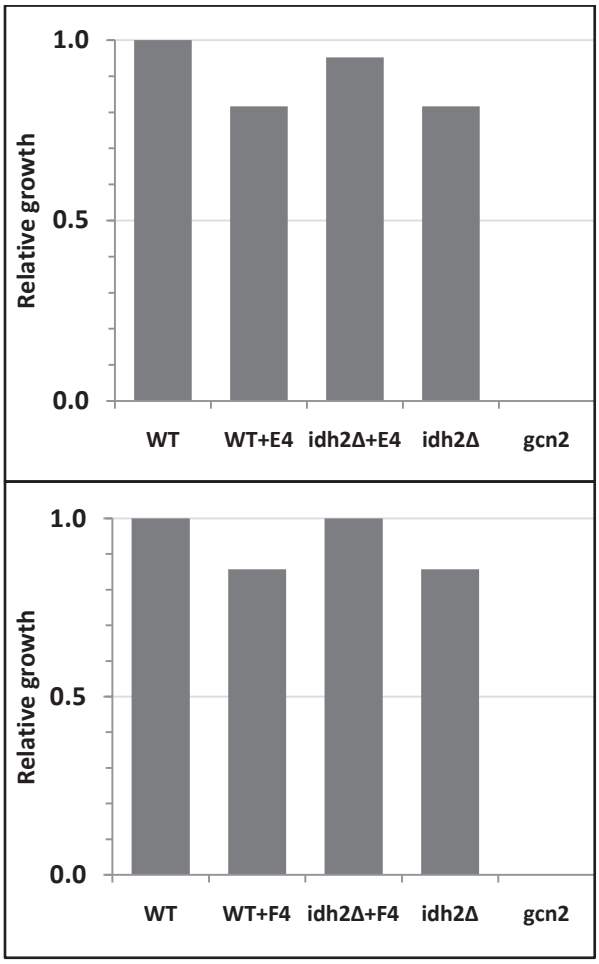


Figure D14: The growth defect of *idh2*Δ strains was restored with a plasmid containing *IDH2*. Quantification of the growth assays was done as in Figure D6 by taking the ratio of SM to SD. E4 and F4 are the two Tiling Collection plasmids.

*D.5.2 idh2*Δ strains showed a strong growth defect when provided a non-fermentable carbon source and this was partially restored by introducing *IDH2*

Idh2 is an isocitrate dehydrogenase subunit and produces NADH, CO_2 and α-ketoglutarate from isocitrate in the citric acid cycle. The other subunit is Idh1 which was identified once in a complex with Yih1 indirectly in the published data and in a complex with both GST-Yih1 and GST-Yih1*H2. It was found that strains deleted for either of the two subunits showed low isocitrate dehydrogenase activity (Cupp and McAlister-Henn, 1991; Zhao and McAlister-Henn, 1996a). These strains grew like the wild type on glucose-containing rich medium but grew slow on glycerol-containing rich medium. This was verified by growing *IDH2* deletion strains on rich medium containing glycerol and this resulted in a strong growth defect compared to the wild type, similar to published research (Figure D15) (Cupp and McAlister-Henn, 1991).

The growth defect for *IDH2* deletion strains on rich glycerol media was partially restored by reintroducing *IDH2* on a plasmid from the Genomic Tiling Collection but not to wild type levels (Figure D13). Thus, a full growth reversal to wild type levels due to the introduction of Idh2 into *IDH2* deletion strains was expected (Cupp and McAlister-Henn, 1991). However, as discussed in the previous section all genes on the plasmids were expressed at high levels and it is likely that the high expression level of *BAG7* may have restricted the growth restoration to some extent.

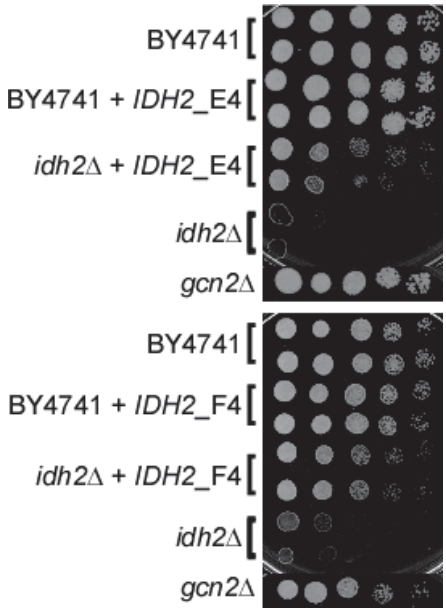


Figure D15: The growth defect of idh2Δ strains in the presence of glycerol (YPG media) was partially restored by plasmid IDH2_E4 and by IDH2_F4. Strains without the plasmid contain an empty vector. Each panel shows the result of a single petri dish.

D.5.3 The growth defect of *idh2*Δ strains is reverted when supplemented with glutamate

As mentioned, *idh1*Δ or *idh2*Δ strains showed a slow growth phenotype on non-fermentable carbon sources but they grew equal to the wild type when provided with the fermentable carbon source glucose (Cupp and McAlister-Henn, 1991; Zhao and McAlister-Henn, 1996a). In the experiments in this work a growth defect for *idh2*Δ strains was seen when supplemented with glycerol but also when glucose was used. Of note is that the growth defect was only observed on minimal medium, indicating that this medium was deficient in nutrients that were necessary for wild type growth.

α-ketoglutarate, the product of isocitrate dehydrogenase activity, is an important precursor for glutamate synthesis. Glutamate is synthesised from α-ketoglutarate and *vice versa* by the glutamate dehydrogenase. It was found that *idh2*Δ strains grew slow on minimal glucose media lacking glutamate (McCammon and McAlister-Henn, 2003). Glutamate synthesis may be impaired in these strains because Idh2 is missing and α-ketoglutarate synthesis is reduced. This may render cells auxotroph for glutamate to some extent and reduce growth due to a lack of protein building blocks. As the minimal medium that was used was missing glutamate this may explain the growth defect seen for *idh2*Δ strains.

NADH is another product of isocitrate dehydrogenase. Without an active enzyme the level of NADH was reduced (Elzinga et al., 1993). Consequently, the level of NAD^+ would increase. In a wild type strain where both Idh1 and Idh2 are present an increase in NAD^+ promotes isocitrate dehydrogenase function. But as Idh2 is missing the cell is unable to resume the citric acid cycle. As NADH is used for ATP production in the oxidative phosphorylation pathway this would reduce energy levels inside the cell. And as the citric acid cycle is inhibited in *idh2*Δ strains this suggests that they instead use fermentation to produce ATP. Wild type strains, on the other hand, may use a mix of both respiratory (via the citric acid cycle) and fermentative metabolism. As the respiratory pathway is more efficient this might confer a growth advantage to wild type strains. Therefore, this would result in a relative slow growth for *idh2*Δ strains.

As a first test to determine if reduced α-ketoglutarate synthesis or low NADH levels are causing the growth defect of *idh2*Δ strains the growth assays above were repeated by supplementing the minimal media with glutamate. The result of a growth assay is shown in Figure D16. Adding glutamate to the media abolished the growth defect of *IDH2* deletion strains under non-starvation conditions and they grew like the wild type. While glutamate synthesis was not restored due to a lack of *IDH2* and α-ketoglutarate, the cells used the supplemented glutamate and this allowed protein synthesis and cellular growth. Alternatively, glutamate was converted into α-ketoglutarate by the glutamate dehydrogenase, compensating for the loss of Idh2 function. This may have allowed the citric acid cycle as well as NADH and ATP production to resume. Thus, this result supported both the idea of reduced α-ketoglutarate synthesis and low energy levels.

The minimal medium used herein contained histidine and methionine. Histidine can be converted into glutamate and methionine can be converted into succinyl-CoA, the next product in the citric acid cycle after α-ketoglutarate. This suggested that these amino acids might compensate for the loss of Idh2 function to some extent but may not increase NADH levels to wild type levels. In turn, this supported the idea that the growth defect of *idh2*Δ strains was largely due to low energy levels inside the cell and less due to reduced α-ketoglutarate synthesis. On the other hand, it is possible that the cytosolic NADP-specific isocitrate dehydrogenase (subunits Idp1 and Idp2) may be able to compensate for a lack of Idh2 or Idh1 (Cupp and McAlister-Henn, 1992). This supported the idea that the growth defect was caused by reduced glutamate synthesis.

Under starvation conditions when glutamate was not supplemented a growth defect was observed for *idh2*Δ strains compared to the wild type (cf. Figure D14). It was possible that this growth defect was caused either by lack of α-ketoglutarate or NADH or both instead of an impaired starvation response. In order to test this, the starvation media was supplemented with glutamate. The growth defect of *idh2*Δ strains was restored to wild type levels under these conditions (Figure D16). This indicated that *idh2*Δ strains were able to overcome the starvation and suggested that the GAAC was active. Again, a lack of α-ketoglutarate or a lack of NADH are possible explanations for the growth defect of *idh2*Δ strains grown under glutamate-deficient starvation conditions.

Transforming the wild type and *idh2*Δ strains with either of the two Tiling Collection plasmids rendered them slow growing under non-starvation and starvation conditions and when glutamate was missing compared to strains transformed with the vector control (cf. Figure D14). This growth defect for strains containing a Tiling Collection plasmid was also seen for media supplemented with glutamate (Figure D16). This supports the idea that overexpression of *BAG7* causes slow growth. Because *idh2*Δ strains with the vector control grew like the wild type and there was no impaired growth under starvation conditions no evaluation in regards to Idh2 complementation can be made from this growth assay. The comparison of minimal and starvation media indicated that transformation with the *IDH2*_E4 plasmid weakly increased growth for the wild type and *idh2*Δ strains while the presence of the *IDH2*_F4 plasmid weakly reduced their growth (Figure D17). This suggested that under starvation conditions with

glutamate supplementation the overexpression of *BAG7* in the wild type resulted in no or only a very weak growth defect. Similarly, there was only a small growth difference between *idh2*Δ strains with the plasmids and with the vector control when comparing minimal and starvation media (Figure D17). This indicated that reintroducing *IDH2* does not strongly restrict or increase growth, presumably because isocitrate dehydrogenase activity was not changed (Cupp and McAlister-Henn, 1991).

To conclude, supplementing *idh2*Δ strains with glutamate restored their growth to wild type levels under both non-starvation and starvation conditions. In addition, while transformation of the wild type and *idh2*Δ strains with the Tiling Collection plasmids resulted in a slow growth this was not exacerbated under starvation conditions.

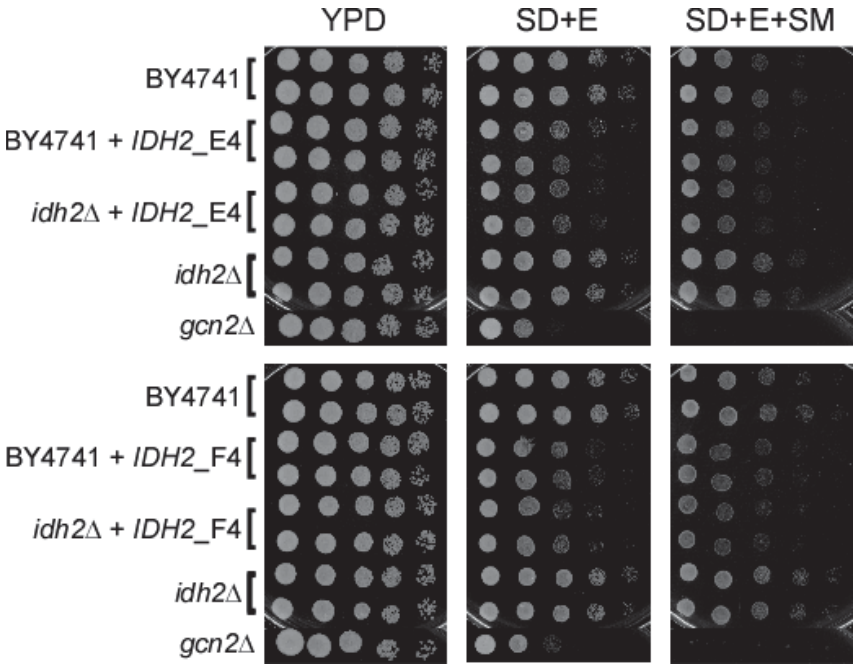


Figure D16: Supplementing *idh2*Δ strains with glutamate abolished their growth defect under starvation conditions. The two plasmids used are *IDH2*_E4 and *IDH2*_F4. E: glutamate. BY4741 is the wild type strain. Strains without the plasmid contain an empty vector. Each panel shows the result of a single petri dish.

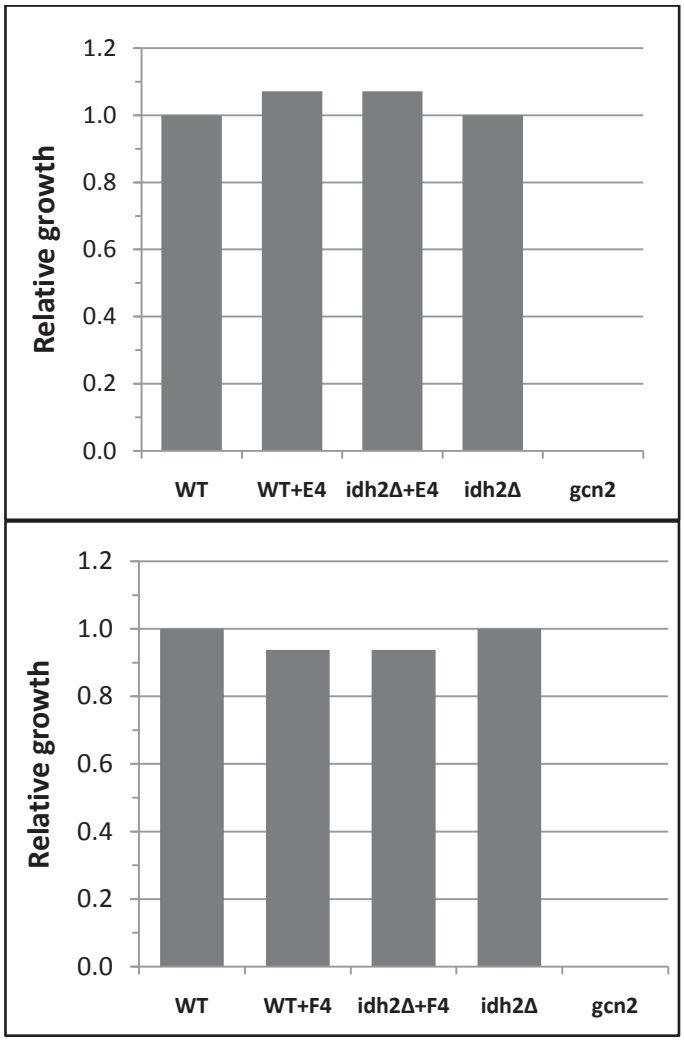


Figure D17: Supplementing *idh2*Δ strains with glutamate abolished their growth defect under starvation conditions. Quantification of the growth assays was done as in Figure D6. The two plasmids used are *IDH2*_E4 and *IDH2*_F4.

D.5.4 Discussion

This study provided evidence that Idh2 may be involved in the starvation response, based on an impaired starvation response in growth assays and reduced eIF2α phosphorylation of *idh2*Δ strains. However, glutamate supplementation restored wild type growth for *idh2*Δ strains in the growth assays. While there were two possible explanations for the growth defect on media lacking glutamate (low α-ketoglutarate levels or low NADH levels), it was not possible to distinguish between them in these experiments. It was conceivable that both are valid and that both contributed to the growth defect, although to an unknown extent.

The enzyme glutamate dehydrogenase converts glutamate to α-ketoglutarate. To further test if the growth defect is caused by low glutamate or low energy levels, a strain deleted for *IDH2* and in addition for the genes encoding glutamate dehydrogenase subunits *GDH1* or *GDH2* could be used. A negative genetic interaction between Idh2 and Gdh2 was already reported, i.e. the double deletion strain had a stronger growth defect than expected from the multiplicative effect of combining two single mutants, indicating a close functional relationship (Szappanos et al., 2011). On media supplemented with glutamate, an *idh2*Δ/*gdh2*Δ double deletion should inhibit the conversion of glutamate into α-ketoglutarate. Consequently, the citric acid cycle should remain inhibited and the cells should grow slow compared to *IDH2* single deletion strains.

To further study the involvement of Idh2 in the starvation response a plasmid containing only *IDH2* but no other protein coding genes needs to be used. This way the growth-inhibitory effects of *BAG7* overexpression can be excluded from the experiment. In addition, it was possible that Bag7 or the gene product of YOR135C (Irc14) may interfere with amino acid biosynthesis or the GAAC. For example, their overexpression could have interfered with Gcn2 activation. Bag7 is involved in actin cytoskeleton organisation and cell wall biosynthesis by stimulating the GTPase Rho1 (Roumanie et al., 2001; Schmidt et al., 2002). Interestingly, Rho1 has been implicated in the control of bud growth (Cabib et al., 1998; Schmidt and Hall, 1998). In addition, overexpression of Bag7 rendered cells sensitive to rapamycin, a drug that inhibits the TOR stress-response pathway (Butcher et al., 2006). One study found that *RHO1* expression was decreased under SM-induced starvation conditions, suggesting that bud growth was reduced to preferably synthesise proteins involved in the starvation response (Jia et al., 2000). Taking into account that the cytoskeleton may regulate Gcn2 activity and that Gcn2 was proposed to be inhibited by Yih1 in the bud (Sattlegger et al., 2004) this may suggest a connection between Bag7/Rho1 and the GAAC. However, no further evidence for such a connection exists in the literature and neither Bag7 nor Rho1 were identified in the Yih1 interactome. Strains deleted for *BAG7* or *RHO1* were not tested for SM sensitivity in the large-scale study by Parsons et al. (2004). Based on these findings it is conceivable that Bag7 overexpression may interfere with Gcn2 activation and this supports the idea that a plasmid only containing the *IDH2* gene would be needed. Not much is known about the function of YOR135C

but it may be involved in DNA metabolism (Alvaro et al., 2007). A *YOR135C* deletion mutant was tested negative for SM sensitivity, suggesting that it is not involved in Gcn2 activation (Parsons et al., 2004).

A separate approach to study the connection between Idh2 and the GAAC is to use other starvation-inducing drugs instead of SM such as 3AT (causes histidine starvation) or L-methionine-S-sulfoximine (MSX, causes glutamine starvation). Yeast strains that cannot activate Gcn2 were unable to grow under such conditions (Cambiaghi et al., 2014; Rolfes and Hinnebusch, 1993). If Idh2 is involved in the starvation response then cells without Idh2 should grow slow on medium supplemented with either 3AT or MSX. This growth defect should then be restored to wild type levels by reintroducing *IDH2* on a plasmid.

In order to further elucidate the role of Idh2, strains deleted for *IDH1*, the other isocitrate dehydrogenase subunit, could be used as this resulted in a similar growth defect as for *idh2*Δ strains (Cupp and McAlister-Henn, 1992). Thus, growing *idh1*Δ under starvation conditions should result in impaired growth and this should be complemented with glutamate supplementation in a similar way as for *idh2*Δ strains. Furthermore, an *idh1*Δ/*idh2*Δ double deletion strain may be used which should also result in a growth defect. There is some evidence that a double deletion may exacerbate the growth defect under certain conditions such as when they were provided with non-fermentable carbon sources (Cupp and McAlister-Henn, 1992; Zhao and McAlister-Henn, 1996b). Thus, it may be possible that this increased growth defect is also seen under starvation conditions.

D.6 Yih1 is in a complex with elongation factor eEF1A

Elongation factor eEF1A was identified in the Yih1 interactome. It co-precipitated more than once with Yih1 in the published studies (Krogan et al., 2006) and co-precipitated with both GST-Yih1 and GST-Yih1*H2 (cf. Figure C19). Furthermore, affinity purification experiments have shown that it may interact with Yih1 (cf. Figure C14, Figure C15, and Sattlegger and Castilho, unpublished data).

eEF1A is a protein with a wide range of functions (Mateyak and Kinzy, 2010). It is a ribosome-binding protein and delivers aminoacyl-tRNAs to the ribosome during translation (Morikawa et al., 1978). eEF1A was found to co-precipitate Gcn2 *in vivo* and *in vitro* (Visweswaraiah et al., 2011b). The

interaction between eEF1A and Gcn2 was reduced under starvation conditions *in vivo* and when uncharged tRNA was present *in vitro* (Visweswaraiah et al., 2011b). It was proposed that eEF1A may deliver the uncharged tRNAs to Gcn2 or alternatively this is mediated by Gcn1 (Visweswaraiah et al., 2011b). In addition, eEF1A was found to bind actin but only when not bound to tRNA, suggesting actin and tRNA binding are mutually exclusive (Liu et al., 1996; Munshi et al., 2001). As eEF1A is a putative YBP, as the Gcn1-Yih1 interaction is inhibiting Gcn2 and as Yih1 was found as an actin-binding protein it is possible that Yih1 is involved in this regulatory pathway. As eEF1A is an essential protein strains deleted for eEF1A cannot be used to test for an impaired starvation response. Instead, the possible interaction between Yih1 and eEF1A was investigated.

An attempt was made to construct a strain carrying either GST-Yih1 or GST alone under a galactose-inducible promoter as well as containing either His_6-eEF1A or untagged eEF1A. However, the approach of transforming *GST* or *GST-YIH1* into eEF1A-containing strains was unsuccessful. Instead, two strains were mated, one containing GST-Yih1 or GST (at overexpressed levels) and one containing His_6-eEF1A or untagged eEF1A (expressed at near native levels). In order to verify that both GST and eEF1A proteins were expressed these diploids were grown to exponential phase, cells were lysed using alkaline lysis and proteins were separated via SDS-PAGE. The presence of His, eEF1A and GST was determined using a western blot (Figure D18). A His signal was only seen in His_6-eEF1A-containing samples (*lanes 3 and 4*) while an eEF1A signal was detected in all samples, as expected. The GST-Yih1 and GST alone signals were detected in strains containing untagged eEF1A and His_6-eEF1A, also as expected. This confirmed that the diploid strains expressed eEF1A or His_6-eEF1A and GST-Yih1 or GST at the same time.

A His-pulldown was done using these strains in duplicate to test if Yih1 co-precipitated with eEF1A. After several washing steps to remove unspecific interactors the protein-covered beads were resuspended in loading buffer. The beads were boiled to remove the proteins from the beads, allowing their migration and separation by SDS-PAGE. A western blot was done using antibodies against eEF1A and GST. It was found that boiling the samples resulted in inefficient removal of proteins from the beads in these experiments and subsequently low

signal intensity on the western blot. Instead, imidazole was used to displace His_6-eEF1A and its interaction partners from the beads.

The apparent size of His_6-eEF1A based on an SDS-PAGE is approximately 60 kDa (Visweswaraiah et al., 2011b). A signal corresponding to this molecular weight was detected on the western blot as well as on the Ponceau S stained membrane (Figure D19 top and bottom, *lanes 3-6*, respectively) but not in the samples containing untagged eEF1A (*lanes 1 and 2*). This indicated that eEF1A was specifically precipitated via its His tag and that in comparison untagged eEF1A was not pulldown significantly.

Next the presence of the GST proteins was tested. The anti-GST western blot showed a signal for GST-Yih1 at the expected molecular weight (~64 kDa) in His_6-eEF1A-containing samples (*lanes 5 and 6*) but not in samples containing untagged eEF1A (*lanes 1 and 2*). This indicated that GST-Yih1 co-precipitated specifically with eEF1A and not unspecifically with the beads. This was supported by the observation that GST alone did not co-precipitate with eEF1A (*lanes 3 and 4*), as expected.

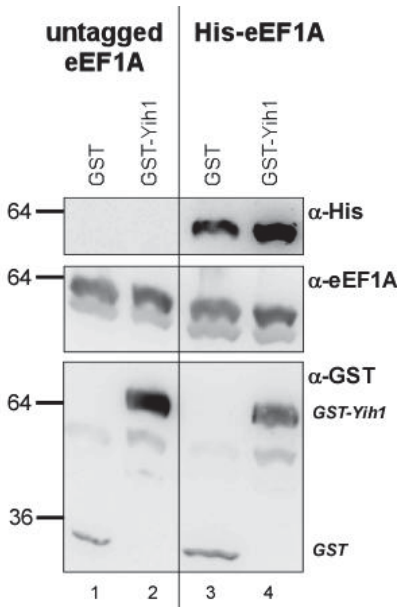


Figure D18: Tagged and untagged eEF1A and GST proteins are expressed in the diploid strains. Western blot was done using antibodies against His, eEF1A and GST.

The membrane was probed with antibodies against Gcn2 as this protein is known to interact with eEF1A (Visweswaraiah et al., 2011b). No Gcn2 signal was detected for samples containing untagged eEF1A (*lanes 1 and 2*), as expected because eEF1A was not pulled down and therefore no Gcn2 should have co-precipitated. A Gcn2 signal was detected for samples containing His_6-eEF1A and GST alone (*lanes 3 and 4*) but in comparison the level of co-precipitated Gcn2 was reduced in samples containing His_6-eEF1A and GST-Yih1 (*lanes 5 and 6*). It was possible that the signal intensities were different because the amounts of Gcn2 protein in the whole cell extract were different. Consequently, less Gcn2 was available for co-precipitation in GST-Yih1-containing samples. For the input control the Gcn2 signal intensity for GST-containing samples was no more than twice the signal intensity that was detected for GST-Yih1-containing samples (*lanes 9 and 10* compared with *lanes 11 and 12*, respectively). For the pulldown samples the Gcn2 signal reduction was around five-fold (*lanes 3 and 4* compared with *lanes 5 and 6*, respectively). Therefore, the Gcn2 signal reduction from GST to GST-Yih1-containing pulldown samples appeared to be much higher than for the Gcn2 input control for the same samples. Thus, the difference in amounts of Gcn2 protein in the whole extract protein alone could not explain the different amounts of Gcn2 that co-precipitated. Alternatively, it was possible that the different amounts of co-precipitated Gcn2 were due to a higher level of His_6-eEF1A in the GST-containing samples. In this case, more His_6-eEF1A would be pulled down and accordingly, more Gcn2 would co-precipitate with His_6-eEF1A. The input signal intensity for His_6-eEF1A in GST-Yih1-expressing samples (*lanes 11 and 12*) was slightly reduced compared to GST-expressing samples (*lanes 9 and 10*). This indicated a low difference in His_6-eEF1A protein levels. Similarly, the pulldown signals showed a low difference in signal intensities, too (*lanes 3 and 4* compared with *lanes 5 and 6*). This indicated that His_6-eEF1A was equally well co-precipitated from samples that contain either GST alone or GST-Yih1. Therefore, samples containing His_6-eEF1A and GST alone co-precipitated more Gcn2 than samples containing His_6-eEF1A and GST-Yih1.

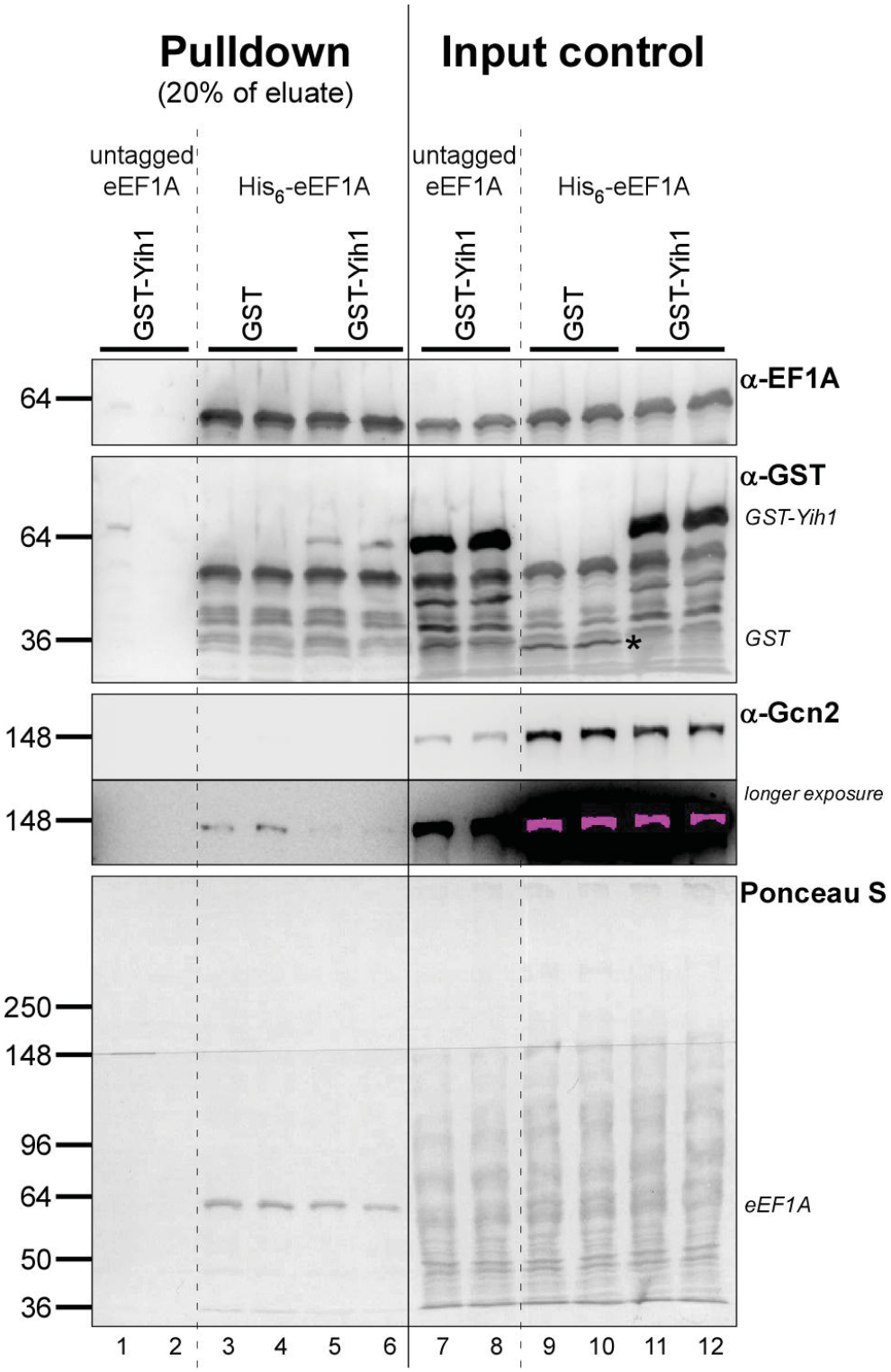


Figure D19: Yih1 was found in a complex with eEF1A. Cells containing His-eEF1A and EF1A and overexpressing either GST and GST-Yih1 were grown to exponential phase, harvested and 1 mg of whole cell extract were used for a His pulldown. A western blot using antibodies against eEF1A, GST and Gcn2 was performed as well as a Ponceau S staining. The GST-Yih1 signal in lane 1 was due to a sample spillover from a lane on the left which is not shown here. Two exposure times are shown for the anti-Gcn2 western blot. In the anti-GST western blot the signal in lanes 3 to 12 and below 64 kDa corresponds to anti-eEF1A background and other smaller bands correspond to GST degradation products. The GST signal in the input control (*lanes 9 and 10*) is indicated by an asterisk.

One explanation for this discrepancy may involve the overexpression of GST-Yih1. GST-Yih1 was more highly expressed than GST alone (compare *lanes 11 and 12* with *lanes 9 and 10*, respectively). Thus, it may have shown an increased level of unspecific binding to other proteins. The overexpression of Yih1 may have driven an unspecific interaction either with eEF1A, especially considering eEF1A is highly abundant itself and showed unspecific interactions (cf. Figure C14Figure C14), or with other proteins in the co-precipitated complex. Thus, a high level of unspecific Yih1-eEF1A interaction may interfere to some extent with the specific Gcn2-eEF1A interaction and displace Gcn2 from eEF1A. This would reduce the amount of Gcn2 that was co-precipitated. It needs to be noted that this does not exclude the possibility that the Yih1-eEF1A interaction is specific but this could not be conclusively determined from this experiment.

To conclude, GST-Yih1 was found specifically in a complex with His_6-eEF1A, suggesting that there is an interaction between the two proteins. An eEF1A-Yih1 interaction is further supported by results presented in Chapter C in which GST-Yih1 co-precipitated eEF1A in a pulldown and by unpublished data by Evelyn Sattlegger and Beatriz A. Castilho.

Discussion

Yih1 was co-precipitating with eEF1A, suggesting that these proteins might interact. If so, then this opens up the possibility that their interaction may affect Gcn2 activity. This idea results from the observations that eEF1A restricts Gcn2 function under replete conditions and allows Gcn2 activity under starvation conditions (Visweswaraiah et al., 2011b). Because Yih1 is a Gcn2-inhibitory protein it may be possible that Yih1-eEF1A interaction stabilises Gcn2-eEF1A interaction to ensure continued Gcn2 inhibition. Another possibility is as follows: Both Yih1 and eEF1A are actin-binding proteins. Therefore, eEF1A may signal actin to release Yih1 and this allows Gcn2 inhibition (Visweswaraiah, 2011). However, future studies are needed to elucidate the eEF1A-Yih1 interaction and its role in Gcn2 activity.

It is important to note that the diploid strains used in these experiments contain a chromosomally expressed eEF1A. This is because two different strains were mated: The haploid strain that only contains His_6-eEF1A was chromosomally

deleted for the two genes encoding for eEF1A (*TEF1* and *TEF2*) but they are still present in the haploid GST-Yih1- and GST-containing strains. For the *in vivo* pulldown experiments a haploid strain with deletions of *TEF1* and *TEF2* instead of the heterozygous diploid would be preferred; however, an attempt at constructing such a strain was unsuccessful. The presence of both native and plasmid-borne eEF1A might have affected the co-precipitation of Yih1 (and Gcn2). Firstly, the relative expression levels of eEF1A and His_6-eEF1A inside these strains is unknown. His_6-eEF1A is expressed from the high-copy plasmid under its own promoter (Visweswaraiah et al., 2011b). This may suggest that native and His_6-eEF1A were expressed at about equal levels. However, as His_6-eEF1A is taken from its genomic context the plasmid may be missing regulatory sequences outside of the promoter that can enhance or decrease expression. The results shown in this work indicate a sufficiently high expression of all types of eEF1A (Figure D19, *lanes 7-12*) but it was not possible to differentiate between them in the input. On the other hand, the eEF1A signal that were detected in the pulldown samples (i.e. *lanes 3-6*) should only contain His_6-eEF1A. This is supported by the observation that an eEF1A signal for samples containing only untagged eEF1A was not detected (Figure D19, *lanes 1 and 2*). This also suggested that His_6-eEF1A expression level was sufficiently high and suggested that the levels of His_6-eEF1A were sufficient for co-precipitation of Yih1. It is possible that the cells preferably expressed one eEF1A version over the other but this was not possible to determine in this experiment.

The number of Gcn2 molecules is relatively low compared to eEF1A and overexpressed GST-Yih1 (Ghaemmaghami et al., 2003). Thus, small changes in the amounts of eEF1A or His_6-eEF1A could result in large differences in Gcn2 that co-precipitated. Gcn2 probably can bind to both native and tagged eEF1A but one needs to consider that Gcn2 could have different binding affinities to each eEF1A type. Even though Gcn2 co-precipitated with His_6-eEF1A, a fraction might have been bound to native eEF1A. As only His-tagged eEF1A was pulled down this would reduce the amount of Gcn2 that was available for co-precipitation. This reduction would be exacerbated if more native than tagged eEF1A were present inside the cell or if Gcn2 preferably bound to native eEF1A. The same applies to Yih1. Based on the western blot results Yih1 may bind to eEF1A or this interaction

might be bridged by another protein (Protein X). Similar to Gcn2, Yih1 or Protein X could associate with native and His_6-eEF1A but with different binding preferences.

eEF1A was tagged with a hexahistidine (His_6) tag. This tag has a relatively low metabolic burden on the cell due to its small size of around 1 kDa (Terpe, 2003; Waugh, 2005). In addition, a glycine linker was inserted between the His tag and the protein which reduces negative effects on protein activity (Sabourin et al., 2012). Nonetheless, this always depends on the individual protein and there is always a possibility that a tag affects protein function. Furthermore, the location of a tag can affect its function. One study found that attaching the His tag N-terminally to lactate dehydrogenase from bacteria resulted in a protein that functioned like the untagged protein (Halliwell et al., 2001). A tag at the C-terminus, however, resulted in an enzyme with lower activity. This was supported by other studies which found that tagging at the C-terminus reduced protein function (Freydank et al., 2008; Perron-Savard et al., 2005; Sabaty et al., 2013). There was insufficient evidence that tagging a protein at the N-terminus resulted in a similar level of reduced protein activity (Freydank et al., 2008; Halliwell et al., 2001; Perron-Savard et al., 2005; Sabaty et al., 2013). In addition, other effects were observed, such as increased binding or increased autophosphorylation (Beitzinger et al., 2012; Dickson et al., 2013; Neumeyer et al., 2006). In this and previous studies the His tag was at the N-terminal end of eEF1A and its function was not affected (Visweswaraiah et al., 2011b). Based on published evidence it is unlikely that His_6-eEF1A activity was reduced, although one study found that N-terminal tagging with the (small) HA tag affected eEF1A function in *Trypanosoma brucei* (Greganova and Bütikofer, 2012). Therefore, it is possible that some eEF1A functions were affected by N-terminal tagging but not others, especially considering that eEF1A has a multitude of functions (Mateyak and Kinzy, 2010). Thus, possible complications resulting from its presence on eEF1A that may interfere with co-precipitating Yih1 or Gcn2 are discussed in the following sections.

It is possible that His_6-eEF1A might not perform its function to the same efficiency or in the same location as untagged eEF1A. For example, its ribosome interaction might be reduced compared to native eEF1A, reducing its ability to bind Gcn2. Previous research has shown that His_6-eEF1A is able to co-precipitate the small ribosomal subunit RPS22, suggesting that ribosome-interaction was not

affected (Visweswaraiah et al., 2011b). However, this was done using haploids strains without *TEF1* and *TEF2* and might not directly apply to this work and there may be functional differences between tagged and native eEF1A. Another possibility is that the His tag may reduce protein binding to the N-terminus of eEF1A. The binding location of Gcn2 in eEF1A is not known yet but an N-terminal His tag may affect their interaction, the putative interaction with Yih1 or other interactions.

It was proposed that Yih1 performs its Gcn2-inhibitory function on the ribosome (Waller et al., 2012). eEF1A likely performs its Gcn2-binding on or near the ribosome (although the eEF1A-Gcn2 interaction itself does not depend on the ribosome) (Visweswaraiah et al., 2011b). If native eEF1A is more likely to interact with ribosomes than His_6-eEF1A and if Yih1 is performing its function on the ribosome then Yih1 may preferably bind native eEF1A instead of His_6-eEF1A. If so, then an eEF1A-Yih1 complex would not be precipitated in this His pulldown and thus this interaction was not detected. Similarly, if Gcn2 binds preferably to eEF1A then this interaction cannot be detected.

If His_6-eEF1A was less likely to bind to the ribosome then this might suggest that the co-precipitated Yih1 was not bound to the ribosome and was not involved in Gcn2 regulation. Therefore, the His_6-eEF1A-Yih1 interaction seen in this work's experiments may not translate into an actual interaction under physiological conditions inside the cell. A ribosome-bound and Gcn2-inhibiting Yih1 may bind to native eEF1A under the tested conditions but this was not possible to be determined from this experiment.

Studies using the protozoa *Tetrahymena* and *Trypanosoma brucei* provided evidence that eEF1A can form dimers and that this was important for its function. While both its monomeric and dimeric form can interact with actin only eEF1A dimers are bundling F-actin (Bunai et al., 2006; Greganova et al., 2010). It is possible that eEF1A dimers occur in yeasts due to the evolutionary conservation of eEF1A. If eEF1A forms dimer in yeast then the presence of His_6-eEF1A might interfere with the dimer formation of native eEF1A, affecting eEF1A function and its interaction with other proteins, i.e. Yih1 and Gcn2. Alternatively, His_6-eEF1A is less likely to form dimers. In either case, if eEF1A dimers are reduced this would reduce its organisation of actin and may affect other cell function due to the importance of actin function. Possibly supporting this, it was found that the His tag

can affect protein dimerisation (Rakowski and Filutowicz, 2013; Wu and Filutowicz, 1999). Thus, it is possible that 1) eEF1A can act as a dimer and that 2) the His tag may affect this dimerisation. If eEF1A can form dimers then this could affect the pulldown in a number of ways. For example, an eEF1A/His_6-eEF1A dimer may not bind and co-precipitate Gcn2 as efficient as an eEF1A monomer or as a dimer consisting only of untagged or His_6-eEF1A. In addition, a reduced level eEF1A dimerisation would affect actin bundling and presumably increase the number of monomeric actin. As Yih1 binds monomeric actin this may affect the amount of Yih1 that is available for co-precipitation.

Using purified GST-Yih1 and His_6-eEF1A would be a first step to alleviate some of these concerns. If Yih1 and eEF1A are directly binding then purified proteins should interact *in vitro*. It would be interesting to test if this can affect eEF1A-Gcn2 interaction. In addition, studies using Yih1 and eEF1A fragments can be used to map the binding locations in each protein, as was done for the Yih1-Gcn1 and Yih1-actin interactions (Sattlegger et al., 2011).

Chapter E

Yih1 and genetic interactions

Chapter E: Yih1 and genetic interactions

The Yih1 interactome consists of physical interactions but the relationship between proteins can also be studied on a functional level by identifying genetic interactions between two proteins. Two proteins are considered to have a genetic interaction when the double mutant shows a stronger or weaker growth defect than expected by the multiplicative effect of the individual single deletions. For example, the two strains with a single deletion each show a reduced but relatively high fitness while the respective double deletion strains cannot survive. It is also possible that the double deletion shows a less severe growth defect than expected.

One large-scale study investigated genetic interactions between two proteins (Costanzo et al., 2010). In total, they evaluated around 1,700 genes for a genetic interaction. This indicated that not all possible genetic interaction were identified. They found that *YIH1* has a genetic interaction with 18 genes, including four that were found at least once with Yih1 in the published interactome (*RTN2*, *SKI3*, *SOL1* and *TPS2*) and one that was found in both published and in-house interactome (*TEF4*, the subunit gamma of elongation factor eEF1B). Only two of the 18 deletion strains have a published SM sensitivity when the respective genes were removed from the cell, *MNN10* and *UME6* (Parsons et al., 2004). The respective proteins of *MNN10* and *UME6* were not found in the Yih1 interactome, suggesting that the Yih1 interactome is still incomplete. *TEF4* deletion strains were tested by the Parsons group but they found no SM^s (Parsons et al., 2004).

A genome-wide chemical-genetic screen used heterozygous diploid yeast deletion strains and grew them at 15˚C, 30˚C and 37˚C in the presence of the drug macbecin II (Franzosa et al., 2011). This drug inhibits proteins that belong to the Hsp90 family of heat shock proteins. Among the heterozygous diploid deletion strains whose growth was negatively affected at 15˚C was *yih1*Δ/*YIH1*. A previous study by the same group grew homozygous diploid deletion strains at 30˚C using macbecin II but they did not identify Yih1 (McClellan et al., 2007).

In yeast, the Hsp90 family consists of the two proteins Hsc82 and Hsp82 (Borkovich et al., 1989). Both were found in the published interactome at least once and in the in-house interactome with both Yih1 and the Yih1 mutant

(cf. Figure C19). This suggested that these proteins may be YBP. *hsc82*Δ was tested for SM sensitivity by Parsons et al. but they found no impaired growth and were therefore not tested in this work. However, the growth defect might not be detectable at the temperature they tested (30˚C) when taking into account the study by McClellan et al. (2007) mentioned above.

A list of genes that showed a genetic interaction with *YIH1* is given in Table E1. None of the genetic interactors was tested in the study by Zhang et al. (2008).

Table E1: A selection of genes that showed a genetic interaction with *YIH1*. *HSC82*, *HSP82* and *TEF4* were identified in the Yih1 interactome. The study by Parsons et al. (2004) that tested the deletion strains for SM sensitivity is given. An empty cell indicates that the protein was not tested. Proteins shaded in grey indicates proteins that were further analysed in this chapter.

Gene	Source	Parsons 2004
MNN10	Costanzo 2010	yes
UME6	Costanzo 2010	yes
HSC82	Franzosa 2011	no
HSP82	Franzosa 2011	
TEF4	Costanzo 2010	no
EEG2	Costanzo 2010	no
LSM6	Costanzo 2010	no
MFB1	Costanzo 2010	
MSC1	Costanzo 2010	
PEX8	Costanzo 2010	
PKR1	Costanzo 2010	no
PSH1	Costanzo 2010	
RPS21B	Costanzo 2010	
RPS27B	Costanzo 2010	
RTN2	Costanzo 2010	
SKI3	Costanzo 2010	
SOLI1	Costanzo 2010	
TPS2	Costanzo 2010	no
UBC6	Costanzo 2010	
YNP5	Costanzo 2010	no

E.1 Both Mnn1 and Ume6 may not be involved in promoting Gcn2 activity

Similar to Chapter D the published SM sensitivity for *ume6*Δ and *mnn10*Δ deletion strains was verified. This was done by comparing their growth under SM-induced starvation conditions and was followed by measuring the level of eIF2α-P as an indicator of Gcn2 activity (cf. Chapters B.11 and B.15).

*mnn10*Δ strains showed a strong growth defect under starvation conditions compared to the wild type while *ume6*Δ strains did not (Figure E1, *A*). In addition, deletion of *MNN10* did not significantly change eIF2α-P levels (*B*). This suggested that both Ume6 and Mnn10 are not necessary for promoting Gcn2 activity. However, Ume6 has a connection to the putative Yih1-binding protein Spc72: At the spindle pole body Spc72 is replaced at the end of meiosis I by two proteins, Mpc54 and Spo21. Interestingly, expression *MPC54* and *SPO21* is positively regulated by Ume6. As Spc72 is a strong candidate for a positive Gcn2 regulation it might be interesting to study the role of Ume6 in this context. Ume6 is a transcriptional regulator during growth and meiosis. Notably, it was shown that it is important in regulating the cell's response to low nitrogen conditions (Bartholomew et al., 2012).

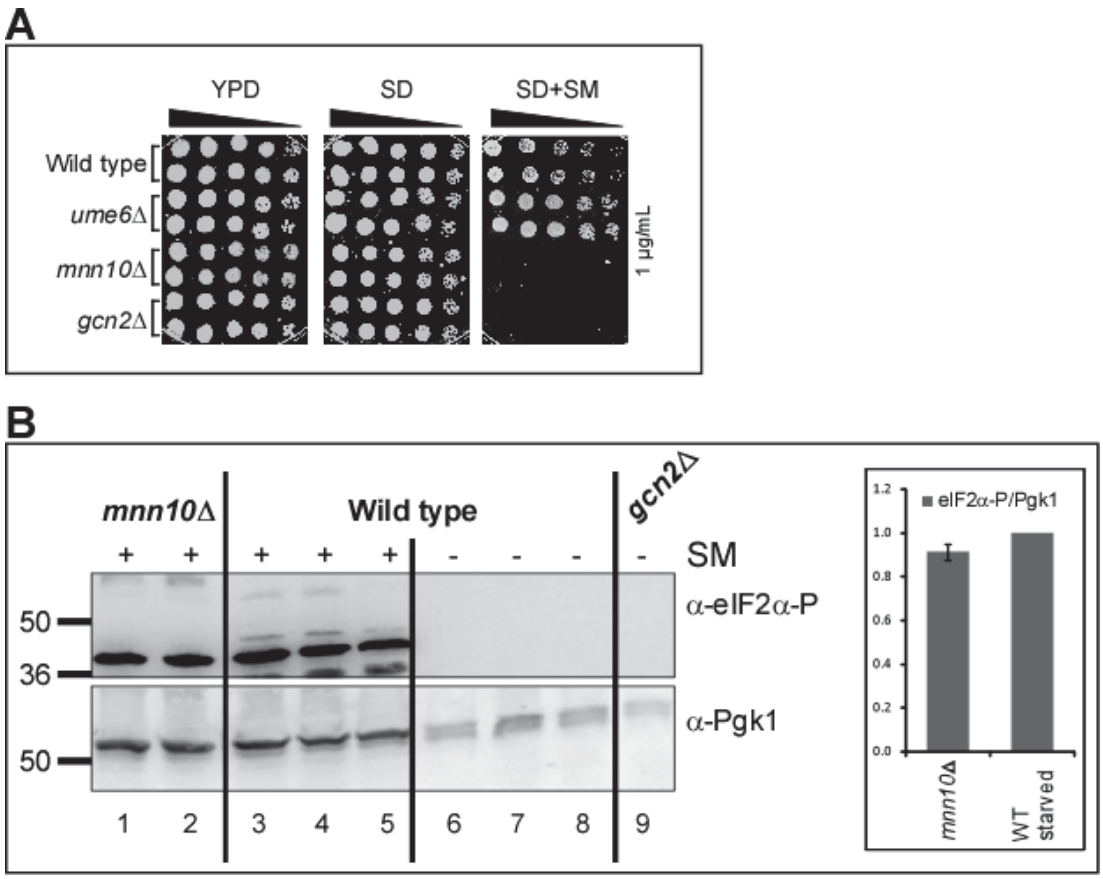


Figure E1: *mnn10*Δ strains were SM sensitive (A) but showed no significantly reduced Gcn2 activity (B). *ume6*Δ strains were not SM sensitive. Starvation experiments and western blot were done as described previously in Chapter D. WT: Wild type.

E.2 The SM sensitivity of *HSC82* deletion strains is reversed by deleting *YIH1*

A growth assay for *hsc82*Δ and *hsp82*Δ strains was performed to investigate its SM sensitivity. In addition to the growth assay at 30˚C, both strains were tested at 15˚C because the *YIH1-HSC82* genetic interaction was found at this temperature using heterozygous but not homozygous diploids (Franzosa et al., 2011; McClellan et al., 2007). In addition, as *HSC82* is moderately activated under heat stress (Borkovich et al., 1989) 37˚C were included in these assays. For *hsc82*Δ strains growing under starvation conditions a moderate growth defect at 30˚C, a strong growth defect 37˚C and no growth defect at 15˚C was found compared to the wild type (Figure E2). *hsc82*Δ strains did not show a growth defect at 37˚C on control medium, likely because the loss of *HSC82* was complemented by Hsp82 under these conditions (Borkovich et al., 1989). This suggests that cells without Hsc82 are unable to activate the starvation response. Furthermore, the strong growth defect at 37˚C indicates that, under starvation

conditions, *HSC82* deletion could not be complemented by Hsp82. No growth defect was observed at 15˚C and this was in contrast to the published findings (Franzosa et al., 2011). This suggested that Hsc82 is not important to activate the starvation response at this temperature.

The next question was if this growth defect is mediated via Yih1. If so, then deletion of *YIH1* in *hsc82*Δ strains should restore growth to wild type levels. To this end, a strain was deleted for both *YIH1* and *HSC82* as described previously for *SPC72* in Chapter D.4 (see also Appendix C for verification) and the growth assay was repeated. These *hsc82*Δ/*yih1*Δ double deletion strains grew like the wild type under starvation conditions (Figure E2, “vector”), indicating that the deletion of *YIH1* restored growth. This suggested that Hsc82 may have an inhibitory effect on Yih1 function such as inhibition of the starvation response by reducing Gcn2 activity. Thus, removing Hsc82 from the cell could free Yih1 and allow Gcn2 inhibition. Subsequently, if Yih1 is removed in cells without Hsc82 then Gcn2 is free to be activated and this would explain why the growth defect was reverted. If that is the case then reintroducing *YIH1* into *hsc82*Δ strains should result in Gcn2 inhibition. To test this, the *hsc82*Δ/*yih1*Δ double deletion strain was transformed with *YIH1* that was tagged with the Venus fluorescence protein and that was expressed at native levels (VN-Yih1, cf. Table B3). This should result in an impaired growth as was seen for *hsc82*Δ strains. However, no growth defect was seen and they grew equally to the *hsc82*Δ/*yih1*Δ double deletion strains and the wild type (Figure E2, “Yih1”). This suggested that cells are able to activate the starvation response and that Yih1 does not inhibit Gcn2, contrary to expectations. However, because this strain grew as if Yih1 was missing it was possible that the tag affected Yih1 function. Another possibility was that Yih1 and Hsc82 interaction is only needed under certain conditions. Additionally, a successful double deletion was inferred from the complementation of selectable markers and not confirmed by PCR (Appendix C). Therefore, the reversion of the growth defect seen in Figure E2 may be a result of, for example, secondary mutations that enabled the cell to overcome the starvation instead of a deletion of both *HSC82 and YIH1*.

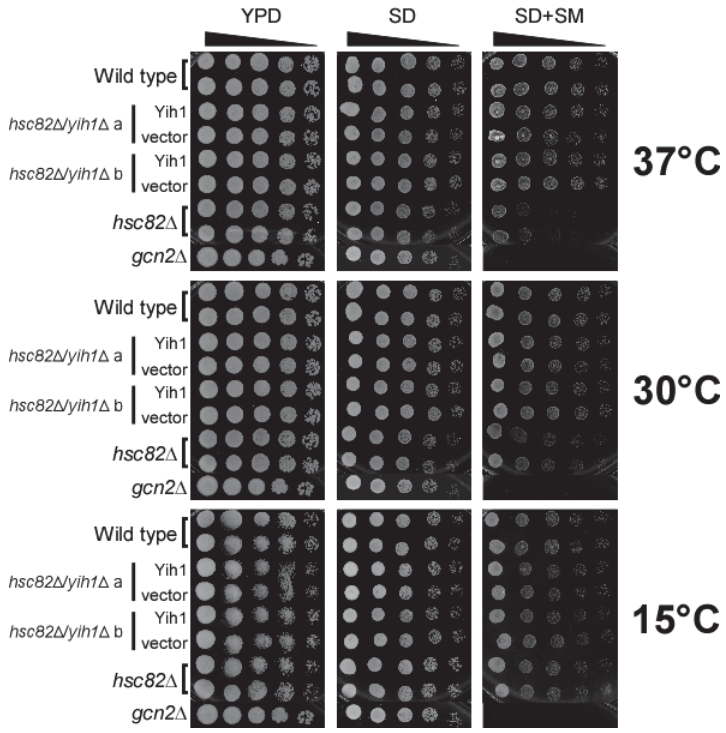


Figure E2: *hsc82*Δ strains were SM sensitive at 30˚C and 37˚C and this growth defect was restored by deleting *YIH1* in these strains ("vector"). However, transforming *hsc82*Δ/*yih1*Δ double deletion strains with a Yih1 that was tagged with the N-terminal fragment of the Venus fluorescence protein (plasmid pRR04a, provided by Rashmi Ramesh) did not result in the expected growth defect (a, b: independent colonies). No growth defect was observed at 15˚C. Starvation experiments and western blot were done as described previously in Chapter D. Each panel shows the result of a single petri dish.

E.3 The SM sensitivity of *TEF4* deletion strains is not reversed by deleting *YIH1*

A growth assay for *TEF4* deletion strains was performed, as described above for *HSC82* deletion strains. *tef4*Δ strains showed a growth defect at 30˚C and almost no growth at 37˚C under starvation conditions (Figure E3). This was in contrast to the study by Parsons et al (2004) which showed no growth defect. There was a small growth defect for *tef4*Δ strains at 15˚C; however, a similar growth defect was seen on the SD control plate. A *tef4*Δ/*yih1Δ* double deletion strain was constructed but their growth defect was similar as seen for the *TEF4* deletion strains. This suggested that Gcn2 activity was reduced but that the slow growth phenotype occurred independently of Yih1. However, the possibility that other proteins of the GAAC were involved cannot be excluded. Similar to Hsc82

above the double deletion was inferred based on the complementation of selectable markers (Appendix C) and therefore it was possible that no double deletion was produced.

TEF4 encodes for a non-essential gamma subunit of translational elongation factor eEF1B (Kinzy et al., 1994). eEF1Bγ and another protein, eEF1Bα, form a complex and together they are the guanosine-exchange factors that regenerate the inactive GDP-eEF1A and allow another round of charged tRNA-binding. eEF1Bγ contains HEAT repeats and the presence of regions with similarity to HEAT repeats in Gcn1 and Gcn20 suggested that they have a similar function as eEF1Bγ (Marton et al., 1997). eEF3 is competing with Gcn1 for ribosome binding and this results in an inhibition of the GAAC (Visweswaraiah et al., 2012). In addition, eEF1Bα and actin bind in the same region in eEF1A and therefore eEF1Bα can prevent actin-mediated inhibition of eEF1A, thereby guiding eEF1A to protein translation (Pittman et al., 2009). The possible scenario for Yih1 regulation by eEF1Bγ is not obvious, especially considering that a *tef4*Δ/*yih1*Δ double deletion did not restore the growth defect of *tef4*Δ strains. Since eEF1B and eEF1A are closely linked it may be possible that Tef4 (or eEF3) co-precipitated with Yih1 via eEF1A or actin. Thus, while it may play a role in Gcn2 regulation this may not involve Yih1.

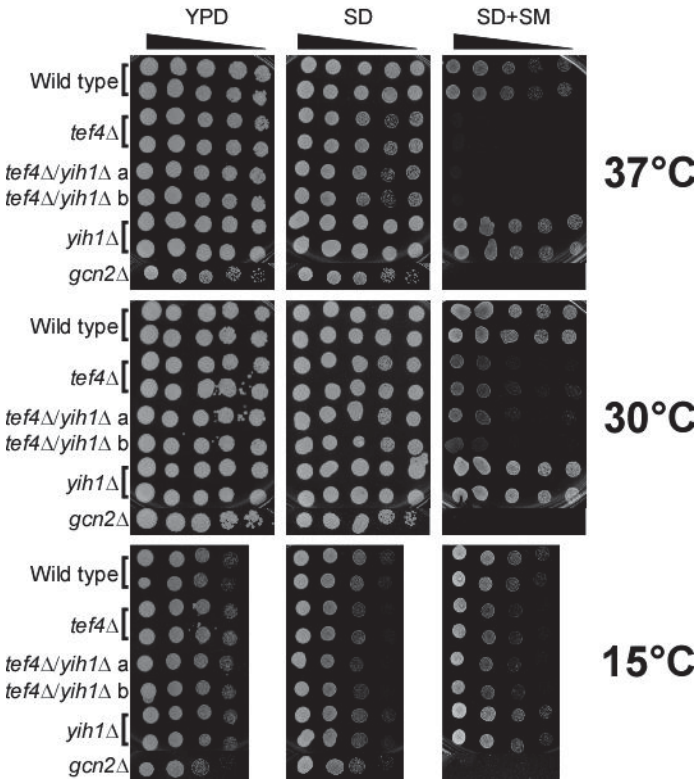


Figure E3: *tef4*Δ strains were SM sensitive at 30˚C and 37˚C but this growth defect was not restored by deleting *YIH1* in these strains (a, b: independent colonies). The growth defect observed at 15˚C on SM-containing media was also seen on SD control media. For 15˚C the fifth dilution column was removed in this image due to erroneous cell transfer. Each panel shows the result of a single petri dish.

Chapter F

Conclusions

Chapter F: Conclusions

This study aimed to advance our knowledge about novel proteins binding to Yih1 and how this interaction affects Gcn2 activity. For example, the spindle pole body protein Spc72 was identified as a potential YBP in this work and it was found to be necessary for Gcn2 activation. But how is Spc72 regulating Gcn2 activity? Under starvation conditions cells transition into a reversible state called quiescence or G_0 phase in which no proliferation occurs (De Virgilio, 2012). The cell undergoes a wide variety of internal changes during transition into the G_0 phase to allow cell survival (De Virgilio, 2012). For instance, a recent study found evidence that, in G_0 phase, microtubules localised to the nucleus instead of the cytosol as in dividing cells (Laporte et al., 2013). The physiological function of forming nuclear microtubules is not clear but they may be important for cell survival under starvation (Laporte et al., 2013). These nuclear microtubules originated from and were attached to the spindle pole body and this included Spc72 (Laporte et al., 2013). It is possible that the formation of nuclear microtubules signals the cell to activate Gcn2 and that this is accomplished through Yih1-inhibition by Spc72. Conversely, without Spc72 the formation of nuclear microtubules may be impaired. This idea is supported by the observation that the Spc72 interactor Kar3 and other proteins such as Nip100 were required for the formation of nuclear microtubules (Laporte et al., 2013). It is not clear how deletion of Kar3 or Spc72 results in defective bud formation but it may involve the accumulation of misaligned microtubules (Laporte et al., 2013). Misaligned microtubules due to *SPC72* deletion may be impaired in their ability to inhibit Yih1 which is then free to restrict Gcn2 activity, resulting in an impaired starvation response.

Yih1 has a proposed Gcn2-inhibitory role in the bud tip (Waller et al., 2012) and this may involve Spc72. This idea is based on observations that the spindle pole body is located near the bud neck in order to ensure that daughter and mother cell receive a nucleus during cell division (Schott et al., 2002). Thus, as Spc72 is part of the spindle pole body it is in proximity to the region where Gcn2 needs to be inhibited. In this case, Spc72 would need to release Yih1 to allow Gcn2 inhibition.

One intriguing possibility is that actin is involved in Spc72-facilitated Gcn2 inhibition. In fact, actin-microtubule interactions are important for many fundamental processes inside the cell, including cell division (Goode et al., 2000; Rodriguez et al., 2003). Actin ensures proper cell wall synthesis and directs secretory vesicles and myosin to the growing bud (Schott et al., 2002). In the growing bud, actin was proposed to release Yih1 in order to promote Gcn2 inhibition and a high level of protein synthesis (Waller et al., 2012). The idea of relationship between actin and Spc72 is supported by the observation that *ACT1* has a genetic interaction with *SPC72* (Costanzo et al., 2010). A genetic interaction does not mean physical interaction but nonetheless it informs us about a possible functional relationship. In this case, the functional relationship between Spc72 and actin is interesting because of the known functional relationship between Yih1 and actin. It is possible that Spc72 is part of a crosstalk between microtubules and actin that triggers the release of Yih1 from actin, resulting in Gcn2 inhibition. Conversely, without Spc72, the Yih1-actin interaction is retained and Gcn2 is active in the bud tip, restricting its growth.

During bud emergence the spindle pole body duplicates and one unit is oriented towards the growing bud and the other is oriented towards the cytosol. Spc72 is involved in this duplication process (Jaspersen and Winey, 2004). After duplication, Spc72 may be present on both spindle pole bodies or it may be attached to only one. Thus, Spc72 function may depend on its location. Supporting this idea of an asymmetric function, there is evidence that Spc72 may be preferably recruited to the bud-oriented spindle pole body by the gamma-tubulin complex (Juanes et al., 2013). Therefore, this would ensure that Spc72 is near the bud tip and that Yih1 is active.

How can the cell promote Spc72-mediated Gcn2 inhibition? Spc72 regulation is likely complex as it binds different proteins at different cell cycle stages. For example, it binds Kar1 in G_1 and Nud1 in S phase and Kar3 and Kar1 during mating (Grueneberg et al., 2000; Pereira et al., 1999). In addition, its phosphorylation state changes throughout the cell cycle. For instance, Spc72 is not phosphorylated during G_1 but is phosphorylated by Cdc5 at the G2/S checkpoint or during G2 phase (Grueneberg et al., 2000; Paulson et al., 2008). This results in the attachment of Spc72 to the spindle pole body which allows cell division to continue. Interestingly, Cdc5 was found as a substrate for Cdc28, a

putative YBP (Dautel, 2012; Ubersax et al., 2003). In addition, the Spc72-binding protein Kar1 has been found in a yeast two-hybrid assay as an interacting protein of Cdc28 (Wong et al., 2007). In fact, the purified spindle pole body is highly phosphorylated (Wigge et al., 1998). Thus, it is possible that Cdc5 phosphorylation by Cdc28 allows Cdc5 to phosphorylate Spc72. However, how phosphorylation affects the function of individual components of the spindle pole body is not well known (Jaspersen and Winey, 2004). In addition, the phosphorylation properties of Spc72 under starvation conditions (during G_0 phase) are not known. That said, either phosphorylation or dephosphorylation of Spc72 may allow for Gcn2 inhibition in the bud tip through interaction of Spc72 with Yih1. In turn, this interaction would be broken by either Spc72 de-phosphorylation or phosphorylation.

There is evidence that the phosphorylation level of spindle pole body components increases in response to a stress or a cell cycle checkpoint activation and that this presumably ensures the stability of microtubules (Lin et al., 2011). Thus, it is tempting to speculate that amino acid starvation stress may increase Spc72 phosphorylation. This may ensure Yih1 inhibition, either directly or via another protein such as actin, so that Gcn2 can be active. Consequently, if Spc72 is missing or is not phosphorylated then Yih1 would be active and inhibit Gcn2. Thus, under starvation conditions Spc72 phosphorylation might signal the cell to stop proliferation and start the stress response.

The idea that microtubules may promote GAAC activation under stress conditions is supported by another finding. Benomyl is a drug that destabilises microtubules and we found that *gcn2*Δ or *gcn1*Δ strains were sensitive to benomyl (Cambiaghi et al., 2014). In addition, *yih1*Δ strains were not affected in their growth by benomyl, indicating that Gcn2 was active. Furthermore, overexpression of Yih1 impaired growth on benomyl-containing media, suggesting that Gcn2 function was reduced. One explanation is that, under benomyl-induced stress conditions, microtubule destabilisation may be sensed by Spc72 as this protein is involved in microtubule formation (Chen et al., 1998). Subsequently, Spc72 could signal the cell to stop proliferation and activate Gcn2 via Yih1 inhibition. Conversely, without Gcn2 cells are unable to respond to the microtubule stress. Another explanation is that benomyl-induced microtubule destabilisation may affect the tubulin-mediated transport of amino acids or tRNA (Castilho et al.,

2014). This would result in an increased level of uncharged tRNA and, in turn, this activates Gcn2. Thus, without Gcn2 cells are unable to sense uncharged tRNA and cannot activate the GAAC.

Gcn2 activity is not required under non-starvation conditions. Under these conditions, Spc72 may induce Yih1 activation instead of inhibiting it in order to reduce Gcn2 function. This implies that deletion of Yih1 would render Gcn2 active; however, this is not the case as *yih1*Δ strains were not impaired in their growth on non-starvation media (Sattlegger et al., 2004). Therefore, under non-starvation conditions Spc72-Yih1 interaction may not occur or it is restricted to the bud tip.

The identification of Spc72 as a potential YBP further supported the idea that the cytoskeleton regulates the starvation response. But what is the signal that induces this regulation? Is there a crosstalk between microtubules (via Spc72) and actin? Is Spc72 involved on Gcn2 inhibition under non-starvation conditions and can it activate Yih1 so that Gcn2 is inhibited? Future studies will shed more light on the connection between components of the cytoskeleton and the GAAC.

Idh2 may be involved in the GAAC and Gcn2 activation. Supporting this idea are studies that showed that mitochondrial function, including electron transport involving Idh2 or Idh1, is involved in survival under starvation conditions (Petti et al., 2011). However, the mechanism is not understood yet. Thus, these results open the possibility for a crosstalk between GAAC and mitochondria. There is evidence that supports this connection. For instance, in one large-scale study Idh2 was found to co-precipitate with Gcn1 (Gavin et al., 2006). Idh2 was found in a complex with 13 other tagged proteins and all of these complexes contained eEF1A, an elongation factor that is binding Gcn2. In addition, Gcn1 and Idh2 were found together in 6 out of the 13 complexes. This would support the idea that Idh2 may be involved in regulating Gcn2 activity. On the other hand, Idh2 was not found in a complex with Gcn1 or eEF1A in the large-sale study by Krogan et al. (2006). As discussed in this work not all Yih1-binding are known and one piece of evidence was the low overlap between protein-protein interaction studies. Therefore, this may apply to Idh2 interaction partners as well and does not negate the possibility that Idh2 is involved in the GAAC.

Apart from its function in the citric acid cycle isocitrate dehydrogenase can bind mitochondrial mRNA (Elzinga et al., 1993). However, this function of the

isocitrate dehydrogenase is not well understood yet and its binding to mRNA may only play a subtle role in mitochondrial function (Przybyla-Zawislak et al., 1999). It was suggested that Idh2-mRNA interaction could regulate mitochondrial protein translation (de Jong et al., 2000). Supporting this, it was found that impaired isocitrate dehydrogenase function results in unstable mitochondrial DNA (McCammon and McAlister-Henn, 2003). Thus, this may involve Gcn2, especially considering that Gcn2 activation under amino acid starvation conditions results in an up- or downregulation of the expression of specific mRNAs (Dang Do et al., 2009). However, as mRNA expression is downstream of Gcn2 it is not clear how Idh2-mRNA binding can result in Gcn2 activation. One possibility is that Idh2 activity may not only regulate mRNA but that Idh2 itself is regulated by mRNA. Supporting this, there is evidence that mRNA binding may inhibit Idh2 function (Anderson et al., 2000). Thus, it may be possible that Gcn2 activity regulates the level of Idh2-binding mRNA.

A functional citric acid cycle improves cell survival under starvation conditions (De Virgilio, 2012). Thus, the level of Idh2-inhibitory mRNA should be low. One hypothesis is that an active Gcn2 under starvation conditions reduces the level of inhibitory mRNA, thus allowing Idh2 function. In turn, Idh2 may keep Gcn2 active in positive feedback loop that allows the starvation response, for example through inhibition of Yih1. Conversely, without Idh2, Yih1 is free to inhibit Gcn2 and this would explain the reduced level of eIF2α phosphorylation in *idh2*Δ strains.

Idh2 was found to bind mRNAs encoding for subunits of cytochrome c oxidase, ATP synthase and a small mitochondrial ribosomal protein (Dekker et al., 1992; Papadopoulou et al., 1990). Based on the hypothesis in this work, if these mRNAs are inhibiting Idh2 then, under starvation conditions, their level should decrease upon Gcn2 activation and subsequent Gcn4 activation. One study identified targets of Gcn4 under amino acid starvation in yeast (Natarajan et al., 2001). They found that the expression of genes coding for ribosomal proteins are downregulated, possibly supporting the hypothesis although mitochondrial ribosomes were not discussed by these authors. No increase or decrease in expression level cytochrome c oxidase or ATP synthase was reported. Another study tested mRNA levels in mice liver cells under amino acid starvation (Dang Do et al., 2009). They reported that a number of mRNAs coding for cytochrome c

oxidase subunits, ATP synthase subunits and mitochondrial ribosomal subunits were downregulated by Gcn2. Due to the evolutionarily conservation of Gcn2 and Idh2 function these findings may apply to the yeast system. Therefore, Gcn2 may downregulate Idh2-inhibitory mRNA, thus promoting Idh2 and GAAC activity.

As Idh2 function is needed throughout the cell cycle the Idh2-regulatory pathway that involves Gcn2 and mRNA likely involves other proteins in order to ensure fine-tuning. Alternatively, Gcn2 may upregulate Idh2-activating mRNA. While it is possible that certain mRNA promote Idh2 none have been reported to far. In addition, there may be a competition between inhibitory and activating mRNA for Idh2 binding and this could ensure fine-tuning as well.

A reduced synthesis of glutamate due to *IDH2* deletion may result in an increased level of uncharged $tRNA^{glu}$. This would activate Gcn2 and result in an increased production of amino acids. However, eIF2α-P levels were reduced in *idh2*Δ strains and this indicated a reduced Gcn2 activity. Therefore, the role of Idh2 in the GAAC may be more complex. One explanation is as follows: The level of Idh2-inhibitory mRNA may be reduced upon Gcn2 activation and this would result in a positive feedback loop, ensuring Gcn2 activity. If Idh2 is missing then the level of uncharged $tRNA^{glu}$ may be increased but there would be no Idh2 no ensure continued Gcn2 activity. Consequently, *idh2*Δ strains are unable to phosphorylate eIF2α.

Supplementing *IDH2* deletion strains with glutamate abolished the growth defect of *IDH2* deletion strains grown in media lacking glutamate. Thus, Idh2 may be involved in Gcn2 activation only under certain conditions. When glutamate is present in the starvation medium Idh2 may not be needed for its synthesis. Therefore, it may be possible that Idh2 is involved in the GAAC specifically under glutamate-deficient conditions. In fact, expression of *IDH2* is reduced when glutamate was present (Haselbeck and McAlister-Henn, 1993). Thus, on glucose as a fermentable carbon source and under starvation conditions cells presumably grow as the wild type because Gcn2 was activated.

The identification of Idh2 as a protein that promotes Gcn2 activity opens up the possibility for further studies into the mitochondrial starvation response. Key questions that need to be answered are: What is the mechanism that enables Idh2 to activate Gcn2? Does mRNA binding to Idh2 regulate Idh2 activity in such a way

that Gcn2 remains activated? In order to allow Gcn2 activity, is Yih1 inhibited by Idh2?

The findings in this work showed that Yih1 co-precipitates with eEF1A and that eEF1A can co-precipitate with Yih1. This suggested that eEF1A may be a Yih1-binding protein and that this interaction may be functionally relevant for Gcn2 activation.

How could the interaction between Yih1 and eEF1A regulate Gcn2 function? Studies suggested that under replete conditions Gcn2 activity is reduced when it is bound to eEF1A (Visweswaraiah et al., 2011b). If Yih1 binds eEF1A then it may be possible that this increases and stabilises eEF1A-Gcn2 interaction. Therefore, Gcn2 activity should not increase even when uncharged tRNA is present. However, eEF1A is more abundant than Yih1 and it is not clear how the relatively lowly abundant Yih1 could bind sufficient amounts of eEF1A molecules to allow Gcn2-eEF1A interaction. Furthermore, deletion of Yih1 does not result in increased Gcn2 activity under replete conditions (Sattlegger et al., 2004), indicating that an eEF1A-Yih1 interaction may not be essential for Gcn2 inhibition.

eEF1A may dissociate from Gcn2 under starvation conditions when uncharged tRNA are present and this allows Gcn2 activation (Visweswaraiah et al., 2011b). However, if Yih1-eEF1A is promoting Gcn2 inhibition then how is eEF1A released from Gcn2? This may suggest the existence of other proteins that reduce Yih1-eEF1A interaction, for example actin.

eEF1A may have another role apart from delivering tRNA-delivery to the ribosome. tRNA is able to move between nucleus and cytoplasm in both directions, as was recently discovered, and nutrient starvation can affect this process (Shaheen and Hopper, 2005; Takano et al., 2005). The mechanism is not fully understood yet but evidence from yeast suggested that tRNA moves from the cytoplasm to the nucleus and is then reexported under starvation conditions (Chafe et al., 2011; Pierce et al., 2010). If cells cannot export charged tRNA under starvation conditions they are unable to grow or show a slow growth phenotype (Grosshans et al., 2000; Murthi et al., 2010). Gcn2 was not required for tRNA reexport but the TOR pathway may be specifically involved under nitrogen starvation conditions (Pierce et al., 2010; Whitney et al., 2007). Importantly, eEF1A was implicated in tRNA reexport as mutations in eEF1A near the

tRNA-binding site impaired nuclear tRNA reexport (Grosshans et al., 2000; Murthi et al., 2010). As mentioned, under starvation conditions eEF1A may be removed from Gcn2, allowing the start of the starvation response (Visweswaraiah et al., 2011b). Presumably, eEF1A dissociates from the ribosome due to an unknown signal that may depend on tRNA-binding to Gcn2. eEF1A would now be able to move to the nucleus and promote reexport of charged tRNA, allowing protein synthesis. If eEF1A is involved in reexporting tRNA from the nucleus under starvation conditions then this could be regulated by Yih1. It is not clear how this would occur but there is evidence that Yih1 may be transported between cytosol and nucleus because the Yih1 interactome contains nuclear transporter and proteins present inside the nucleus. That said, this needs to be verified in further studies.

One intriguing possibility is that another elongation factor, eEF3, is involved in GAAC regulation. eEF3 competes with Gcn1 for ribosome binding, thus reducing Gcn1 function and Gcn2 activity (Visweswaraiah et al., 2012). Conversely, under amino acid starvation conditions, eEF3 interaction with the ribosome is reduced and consequently, Gcn1 is able to bind to the ribosome and Gcn2 is activated (Visweswaraiah et al., 2012). It was proposed that under replete conditions eEF1A and eEF3 act together to inhibit Gcn1 and Gcn2 and the starvation response (Visweswaraiah et al., 2012). It is tempting to speculate that Yih1 has a role in this regulatory pathway as it binds Gcn1 and ribosomes, as it was found in a complex with eEF1A and as eEF3 was found in the Yih1 interactome.

The findings from this work and previous unpublished work that indicated that Yih1 may bind to eEF1A raise a number of questions. Is Yih1 directly interacting with eEF1A and what is the effect of their interaction on Gcn2 activity? Does eEF1A-Yih1 interaction stabilise eEF1A-Gcn2 interaction to ensure Gcn2 inhibition? On the other hand, how is a Yih1-eEF1A interaction regulated under starvation conditions when Gcn2 needs to be active? Could actin activate or inhibit Yih1, based on the cellular need, and that this is mediated by eEF1A activity?

Evidence points to an Hsc82-mediated Gcn2 regulation by Yih1. Supporting this, studies found that increased expression of genes encoding for heat shock response is involved in cell survival under starvation conditions (Petti et al., 2011).

One link to further understanding this process may lie in the different isoforms of Hsp90. It was shown that under acetic acid stress conditions Gcn2 activity increased and this was followed by an increased *HSP90* expression as well as increased polysome association of Hsp90 (Silva et al., 2013). Importantly, their findings suggested that the two Hsp90 isoforms Hsp82 and Hsc82 have opposing roles on cell survival under acetic acid stress (Silva et al., 2013): A pro-survival role for Hsc82 and a pro-death role for the Hsp82. This is supported by the observation that Hsp82 is needed for proper Gcn2 maturation (Donze and Picard, 1999) and that Gcn2 was implicated in autophagy (Gentz et al., 2013; Morrison et al., 2013; Tong et al., 2011). This, it may be possible that Hsp82 transmits an autophagy signal to Gcn2 under certain stress conditions. Less is known about Hsc82-Gcn2 interaction but if it has opposing roles to Hsp82 then this suggests that it is a Gcn2 inhibitor. These opposing roles were found under acetic acid stress conditions but they may apply to other stress conditions, considering that Gcn2 is activated by a variety of stresses. For example, experiments from this work showed that the amino acid starvation response of *hsc82*Δ strains was impaired to a higher degree at 37˚C compared to 30˚C.

As Hsp90-binding to the ribosome increased upon Gcn2 activation this allows the possibility that Yih1 is involved. For example, Hsc82 may bind Yih1 and this would allow Hsc82 to bind Gcn2 and perform its inhibitory function. One way to test this would be to investigate whether the Hsc82-Gcn2 interaction is increased if Yih1 is overexpressed in the cell. Conversely, Hsp82 may act as a Gcn2 activator by inhibiting Yih1. Both activation and inhibition may occur on the ribosome. Therefore, the interactions of Hsc82 and Hsp82 with Yih1 that were identified in the Yih1 interactome may be specific. If true, then this suggests that Yih1 can inhibit Gcn2 either via Gcn1 or via Hsp90.

The aim of this study was to find proteins potentially in a complex with Yih1 and that are involved in promoting Gcn2 activity. A number of novel proteins that play a role in the starvation response were identified in this work. This work suggest a complex crosstalk between cellular pathways, indicating that Gcn2 is regulated by signals originating from many locations throughout the cell (Figure F1). However, we are just beginning to understand the role of these novel proteins and their mechanism of promoting Gcn2 activation. In addition, the

findings of this study support the idea of Yih1 as an important player with complex roles in regulating Gcn2 activity. Based on the results from this study, future studies will shed more light on revealing its role in the starvation response.

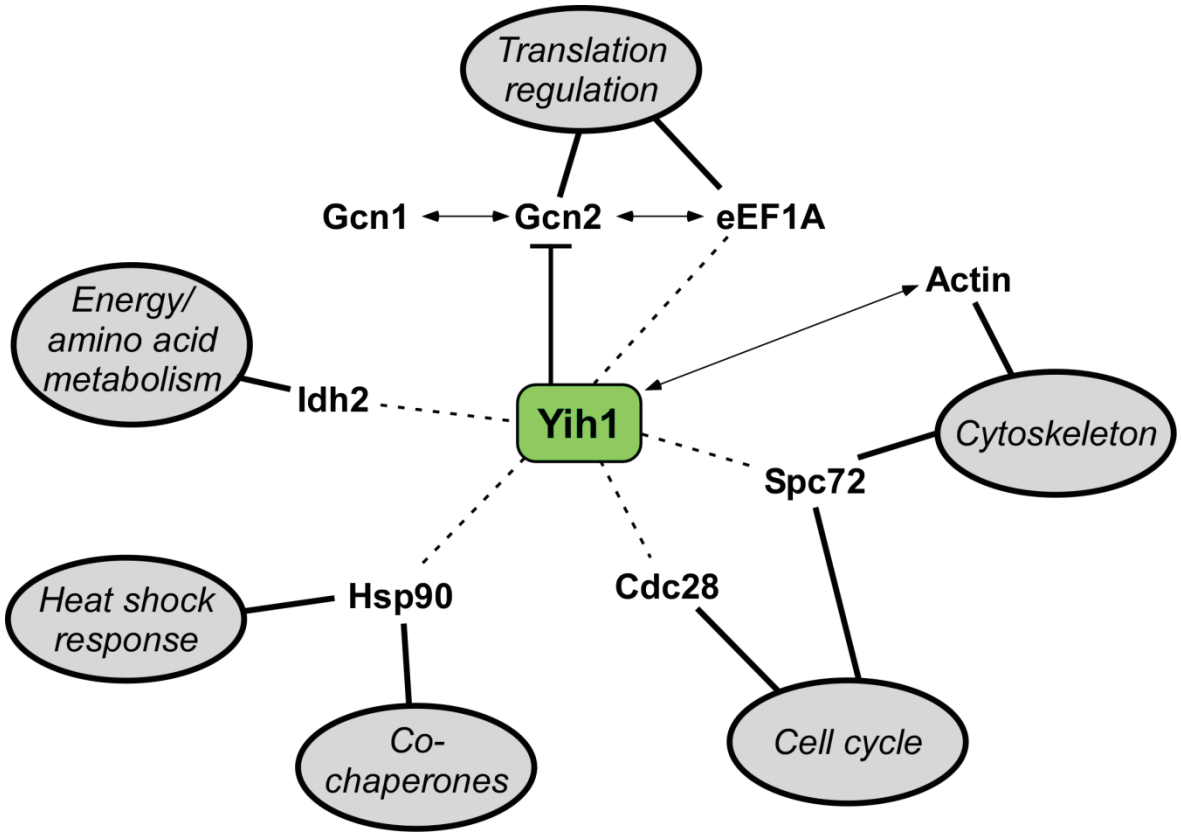


Figure F1: A model of the potential crosstalk between cellular pathways involving Yih1 based on findings from this and previous studies. Double arrows indicate known interactions.

Appendix

Appendix A: Yih1 interactome

The following Yih1 interactome contains all YBP that were identified in the published and in-house studies discussed in Chapter C. For the columns under "Krogan 2006" the scoring is based on the scores in that study, as outlined in Chapter C.1.1. Briefly, "high score" denotes high confidence interactions while "low score" denotes non-significant interactions. As an example, Aap1 ("prey") was found as a low score co-precipitator of seven different proteins ("baits"), including Yih1, and it was only found by the Krogan et al. (2006) study. In another example, Aha1 was found as a low score co-precipitator of three proteins from Krogan et al. and as a significant co-precipitator of the two Yih1 variants from the in-house studies.

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*		TAP				All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
YIH1	YIH1	YIH1	YIH1	YIH1		YIH1	YIH1	YIH1	YIH1	YIH1	YIH1	YIH1	YIH1	YIH1	YIH1	YIH1	YIH1	-
AAC1					AAC1													
AAP1					AAP1	AAP1		AAP1		AAP1	AAP1	AAP1	AAP1					
AAR2										AAR2	AAR2							
ABP1																Abp1		
ABZ1											ABZ1							
ACC1									ACC1		ACC1					Acc1	Acc1	
ACE2								ACE2			ACE2							
ACH1										ACH1						Ach1		
ACO1											ACO1					Aco1		
ACP1											ACP1							
ACS2						ACS2										Acs2		
ACT1																Act1	Act1	
ADE1											ADE01		ADE1					
ADE3																Ade3	Ade3	
ADE4											ADE04		ADE4					
ADE5,7																Ade5,7		
ADE8											ADE8							
ADH1																	Adh1	
ADH3							ADH3											
ADH5											ADH5							
ADK1											ADK1							
ADK2											ADK2		ADK2					
ADR1										ADR1	ADR1							
ADY3					ADY3			ADY3		ADY3								
AEP2											AEP2							
AFG1											AFG1							
AFG3								AFG3			AFG3	AFG3	AFG3					
AGA1											AGA1							
AGE2					AGE2							AGE2						
AHA1										AHA1	AHA1	AHA1				Aha1	Aha1	
AHC2																		
AIR1											AIR1							
ALD4																Ald4		
ALG1								ALG1										
ALG7											ALG7							
ALK1											ALK1							
ALO1																Alo1		
ALR1								ALR1										
AMA1											AMA1							
AME1											AME1							
AMN1											AMN1							
AMS1											AMS1							
ANP1											ANP1							
APA2								APA2		APA2								
APC1					APC1			APC1										
APC11											APC11	APC11						
APC4								APC4				APC4						
APD1								APD1		APD1	APD1		APD1					
APE2											APE2							
APE3																		
APG12											APG12							
APG13											APG13							
APG7										APG7	APG07							
APG9					APG9						APG09							
APJ1													APJ1					
APL2						APL2												
APL3						APL3		APL3	APL3	APL3	APL3		APL3					
APL6											APL6	APL6						
APN2								APN2										
ARA1											ARA1							
ARC40						ARC40												
ARD1									ARD1									
ARF1																Arf1		
ARF3											ARF3							
ARG4											ARG4	ARG4						
ARG5,6							ARG5,6					ARG5,6				Arg5,6		
ARK1											ARK1							
ARL3						ARL3												
ARO1											ARO1					Aro1		
ARO2								ARO2										
ARO3													ARO3					
ARO8	ARO8																	
ARO80											ARO80							
ARP2											ARP2							
ARP3								ARP3			ARP3		ARP3					
ARP4						ARP4												
ART1													ART1					
ARV1											ARV1							
ASE1								ASE1										
ASF2											ASF2							
ASI3											ASI3		ASI3					
ASN1										ASN1								
ASN2					ASN2			ASN2		ASN2	ASN2							
ASP1											ASP1		ASP1					
ASP31											ASP31							
ASP34											ASP34							
AST2					AST2						AST2							

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*			TAP			All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	4 high score ***YIH1***	none **ATP11**	none ***CTL1***	6 high score **ILV1**	8 high score **ILV2**	5 high score **PAT1**	22 high score **Sem1**	4 high score **SHE3**	2 high score **SUI2**	none **VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
ATM1										ATM1	ATM1							
ATP1																Atp1	Atp1	
ATP10													ATP10					
ATP11											ATP11							
ATP12						ATP12		ATP12			ATP12							
ATP2						ATP2										Atp2	Atp2	
ATP3						ATP3										Atp3		
ATP4											ATP4					Atp4	Atp4	
ATP5											ATP5							
ATP7											ATP7							
AVO1					AVO1			AVO1		AVO1	AVO1		AVO1					
AYR1						AYR1										Ayr1		
AZF1												AZF1						
BAP3													BAP3					
BAT1											BAT1							
BAT2						BAT2												
BBC1					BBC1			BBC1		BBC1	BBC1	BBC1				BBC1		
BBP1											BBP1							
BCH1						BDF2												
BCK1													BCK1					
BCK2													BCK2					
BCP1											BCP1							
BCY1											BCY1							
BDF2						BDF2												
BDP1													BDP1					
BEM1										BEM2		BEM2						
BEM2											BEM2		BEM2			Bem2		
BEM3											BEM3		BEM3					
BET4											BET4							
BFA1								BFA1										
BFR1											BFR1							
BFR2						BFR2												
BIK1											BIK1							
BIO3											BIO3							
BMH1																Bmh1		
BMH2								BMH2								Bmh2	Bmh2	
BNI1								BNI1		BNI1	BNI1		BNI1					
BNI5											BNI5							
BNR1								BNR1			BNR1							
BNS1											BNS1							
BOI2										BOI2	BOI2							
BPH1							BPH1				BPH1	BPH1						
BPT1											BPT1							
BRE1											BRE1		BRE1					
BRE2						BRE2					BRE2							
BRO1													BRO1					
BRR2											BRR2		BRR2					
BSP1											BSP1							
BTN2											BTN2							
BUB1							BUB1						BUB1					
BUD13											BUD13							
BUD17											BUD17							
BUD22											BUD22							
BUD3								BUD3										
BUD6											BUD06		BUD6					
BUL2											BUL2							
CAF120											CAF120							
CAF130													CAF130					
CAF16								CAF16					CAF16					
CAF4											CAF4		CAF4					
CAJ1										CAJ1	CAJ1							
CAM1						CAM1		CAM1		CAM1	CAM1							
CAR2	CAR2																	
CBF5													CBF5					
CBK1								CBK1			CBK1	CBK1	CBK1					
CBP1										CBP1	CBP1		CBP1					
CBR1					CBR1			CBR1		CBR1	CBR1	CBR1				CBR1		
CCE1						CCE1		CCE1			CCE1							
CCL1					CCL1	CCL1		CCL1			CCL1	CCL1	CCL1					
CCP1								CCP1		CCP1	CCP1							
CCT2						CCT2		CCT2		CCT2	CCT2							
CCT3																Cct3		
CCT4																Cct4		
CCT6																Cct6		
CCT7																Cct7		
CCT8											CCT8					Cct8		
CCZ1						CCZ1					CCZ1							
CDC1											CDC1							
CDC123																		
CDC19																Cdc19		
CDC2											CDC2							
CDC20													CDC20					
CDC21								CDC21		CDC21	CDC21							
CDC24					CDC24			CDC24										
CDC25					CDC25						CDC25							
CDC31								CDC31										
CDC34						CDC34		CDC34										
CDC39						CDC39				CDC39	CDC39		CDC39					

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*	TAP					All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
CDC4											CDC4		CDC4					
CDC42											CDC42							
CDC43								CDC43										
CDC48											CDC48		CDC48			Cdc48		
CDC53											CDC53	CDC53						
CDC54											CDC54							
CDC73								CDC73		CDC73	CDC73	CDC73						
CDH1											CDH1							
CEM1													CEM1					
CET1								CET1		CET1		CET1						
CFT1						CFT1	CFT1				CFT1							
CFT2										CFT2	CFT2							
CHC1											CHC1					Chc1		
CHD1								CHD1			CHD1							
CHK1								CHK1			CHK1		CHK1					
CHL1										CHL1	CHL1	CHL1	CHL1					
CHL4					CHL4													
CHO2											CHO2							
CHS3					CHS3	CHS3		CHS3		CHS3	CHS3							
CIN8						CIN8				CIN8	CIN8		CIN8					
CIS1											CIS1							
CIT1																Cit1	Cit1	
CKA1											CKA1							
CKA2								CKA2		CKA2								
CLA4								CLA4		CLA4			CLA4					
CLB3											CLB3							
CLC1																		
CLU1					CLU1						CLU1		CLU1			Clu1		
CMD1												CMD1						
CMP2											CMP2							
CNA1										CNA1								
CNE1											CNE1							
COD1										COD1	COD1							
COD2											COD2							
COD5								COD5										
COP1																Cop1		
COQ1																Coq1	Coq1	
COQ2											COQ2							
COR1																Cor1	Cor1	
COS1								COS1										
COS12					COS12													
COS4											COS4							
COS9											COS9							
COX20								COX20		COX20	COX20		COX20					
COX6											COX6							
CPA2																Cpa2		
CPD1											CPD1							
CPR2													CPR2					
CPR3											CPR3							
CPR5								CPR5										
CPR6										CPR6						Cpr6		
CPR7											CPR7							
CPR8											CPR8							
CRM1											CRM1					Crm1		
CRS5											CRS5							
CSE1											CSE1					Cse1		
CSL4							CSL4											
CSM2						CSM2							CSM2					
CSM4											CSM4							
CSR1																Csr1		
CSR2						CSR2		CSR2		CSR2	CSR2							
CTF04					CTF4			CTF4		CTF4	CTF4		CTF4					
CTF13											CTF13							
CTF19						CTF19					CTF19		CTF19					
CTI6					CTI6			CTI6		CTI6	CTI6							
CTK2											CTK2							
CTL1								CTL1										
CTP1								CTP1										
CTR1											CTR1							
CTR9						CTR9							CTR9					
CUP9											CUP9							
CUS2											CUS2							
CVT19								CVT19		CVT19								
CVT9					CVT9					CVT9	CVT9		CVT9					
CWC2											CWC2	CWC2						
CWC21								CWC21										
CWC23											CWC23							
CWC24														CWC24				
CWH41								CWH41			CWH41			CWH41				
CWH43											CWH43							
CYB2											CYB2	CYB2						
CYC2											CYC2							
CYR1											CYR1		CYR1					
CYS4	CYS4							CYS4		CYS4	CYS4							
CYT1																	Cyt1	
DAK1						DAK1					DAK1							
DAK2										DAK2	DAK2							
DAL80											DAL80							

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*		TAP				All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	4 high score ***YIH1***	none **ATP11**	none ***CTL1***	6 high score **ILV1**	8 high score **ILV2**	5 high score **PAT1**	22 high score **Sem1**	4 high score **SHE3**	2 high score **SUI2**	none **VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
DAL81											DAL81							
DAM1											DAM1	DAM1						
DAN1							DAN1											
DBF2													DBF2					
DBP10											DBP10	DBP10	DBP10					
DBP2											DBP2		DBP2					
DBP3											DBP3							
DBP5											DBP5					Dbp5	Dbp5	
DCI1												DCI1						
DCP1											DCP1							
DCS1											DCS1							
DDC1								DDC1			DDC1							
DDP1								DDP1		DDP1								
DED1																Ded1	Ded1	
DEG1											DEG1							
DEP1													DEP1					
DHH1										DHH1								
DIA2					DIA2	DIA2		DIA2		DIA2	DIA2		DIA2					
DIA3								DIA3										
DIN7								DIN7										
DIS3											DIS3		DIS3					
DLD3					DLD3						DLD3							
DMC1													DMC1					
DNA2						DNA2					DNA02							
DNA43								DNA43				DNA43						
DNF1													DNF1					
DNM1																Dnm1		
DOA4											DOA4							
DOM34											DOM34							
DOP1										DOP1	DOP1		DOP1					
DOR1													DOR1					
DOS2								DOS2				DOS2						
DOT5											DOT5							
DPB11										DPB11								
DPM1																Dpm1		
DPS1											DPS1							
DSL1											DSL1							
DST1								DST1		DST1	DST1							
DUF1								DUF1		DUF1	DUF1							
DUN1								DUN1										
DUS3								DUS3			DUS3							
EAF3								EAF3										
EAF5						EAF5		EAF5		EAF5	EAF3		EAF5					
EAF6											EAF5							
EAP1							EAP1											
EBP2											EBP2							
ECI1						ECI1												
ECM16								ECM16			ECM16							
ECM17										ECM17	ECM17							
ECM18								ECM18		ECM18	ECM18		ECM18					
ECM2								ECM2			ECM2							
ECM21											ECM21							
ECM22								ECM22										
ECM25						ECM25		ECM25										
ECM29								ECM29		ECM29	ECM29		ECM29					
ECM30									ECM30	ECM30								
ECM31																		
ECM33																Ecm33		
ECM34											ECM34							
ECM4								ECM4			ECM4							
ECM5								ECM5		ECM5			ECM5					
ECM8											ECM8							
ECO1											ECO1							
EDC2						EDC2												
EFR3						EFR3												
EFT1								EFT1			EFT1					Eft1	Eft1	
EFT2						EFT2		EFT2			EFT2							
EGD1						EGD1			EGD1	EGD1								
EGD2																Egd2	Egd2	
EHD3						EHD3												
EHT1																Eht1		
ELA1					ELA1			ELA1		ELA1	ELA1							
ELP2											ELP2							
ELP3								ELP3			ELP3							
EMP24						EMP24												
EMP70											EMP70							
END3											END3		END3					
ENO2																Eno2	Eno2	
ENT4											ENT4							
EPL1											EPL1		EPL1					
EPS1											EPS1							
ERB1						ERB1												
ERF2											ERF2							
ERG1										ERG1								
ERG11											ERG11	ERG11						
ERG2																		
ERG20					ERG20						ERG20							
ERG27											ERG27							

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*		TAP				All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
ERG5											ERG5							
ERG6						ERG6										Erg6		
ERO1											ERO1							
ERP6								ERP6			ERP6							
ERV2													ERV2					
ERV25											ERV25							
ERV29						ERV29				ERV29	ERV29		ERV29					
ERV46											ERV46		ERV46					
ESA1											ESA1							
ESC1													ESC1					
ESR1					ESR1	ESR1		ESR1		ESR1	ESR1	ESR1						
EST1										EST1								
ETT1																Ett1		
EXO01											EXO1							
EXO70										EXO70	EXO70							
FAA1											FAA1					Faa1	Faa1	
FAA4																Faa4	Faa4	
FAB1						FAB1				FAB1	FAB1							
FAP1								FAB1		FAP1	FAP1		FAP1					
FAR3								FAR3			FAR3	FAR3	FAR3					
FAR7											FAR7							
FAS1																Fas1	Fas1	
FAS2							FAS2				FAS2					Fas2	Fas2	
FAT1											FAT1							
FCY1											FCY1							
FET4											FET4							
FHL1											FHL1							
FIN1								FIN1		FIN1			FIN1					
FIP1								FIP1			FIP1		FIP1					
FIS1											FIS1							
FKH1											FKH1							
FKS1											FKS3					Fks1		
FKS3								FKS3										
FLX1								FLX1			FLX1							
FMP21																		
FOL1						FOL1		FOL1		FOL1	FOL1	FOL1	FOL1					
FOL3											FOL3							
FOX2											FOX2		FOX2					
FRE4											FRE4							
FRE5													FRE5					
FRM2													FRM2					
FRS1											FRS1							
FUB1																		
FUN11								FUN11										
FUN19										FUN19	FUN19							
FUN21											FUN21							
FUN30											FUN30							
FUS1													FUS1					
FYV8										FYV8								
FYV9											FYV9							
FZO1								FZO1		FZO1	FZO1	FZO1	FZO1					
GAA1					GAA1													
GAC1											GAC1							
GAL1													GAL1			Gal1	Gal1	
GAL10																Gal10		
GAL11											GAL11							
GAL7					GAL7							GAL7				Gal7		
GAL80																Gal80		
GAS1											GAS1							
GBP2								GBP2		GBP2								
GCD2													GCD2					
GCD6											GCD6							
GCD7													GCD7					
GCN1								GCN1		GCN1	GCN1	GCN1	GCN1			GCN1		
GCN2								GCN2			GCN2		GCN2					
GCN20											GCN20							
GCN3					GCN3					GCN3	GCN3		GCN3					
GCN5											GCN5							
GCS1											GCS1					Gcs1		
GCV2												GCV2						
GCY1												GCY1						
GDA1											GDA1							
GDH1										GDH1	GDH1					Gdh1	Gdh1	
GDH2							GDH2		GDH2									
GDH3											GDH3							
GEA1											GEA1		GEA1					
GFA1													GFA1			Gfa1		
GFD1								GFD1										
GGA1											GGA1							
GIC1											GIC1							
GIC2											GIC2							
GIN4						GIN4				GIN4	GIN4		GIN4					
GIP2										GIP2			GIP2					
GIR2	GIR2																	
GIS2										GIS2		GIS2	GIS2					
GIS4											GIS4							
GLC7								GLC7		GLC7								
GLN1	Gln1																	

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*										*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		TAP						All baits are low score (<0.02)				TAP			
					includes low-scored interactions, high score are underlined						italics: also as YBP-BP							
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
GLN4											GLN4							
GLO1											GLO1							
GLO3											GLO3							
GLT1								GLT1			GLT1					Glt1		
GND1													GND1			Gnd1	Gnd1	
GPB2											GPB2							
GPI8										GPI8	GPI8							
GPM1																Gpm1		
GPM2													GPM2					
GPT2								GPT2		GPT2	GPT2		GPT2					
GRC3											GRC3							
GRE3										GRE3	GRE3							
GRR1											GRR1							
GRS1											GRS1							
GRX1					GRX1								GRX1					
GRX3																Grx3		
GSC2													GSC2					
GSG1											GSG1							
GSH1											GSH1							
GSY1											GSY1							
GSY2																Gsy2		
GUK1											GUK1		GUK1					
GUS1																Gus1	Gus1	
GUT1											GUT1							
GUT2																Gut2		
GVP36								GVP36								Gvp36	Gvp36	
HAL1								HAL1			HAL1							
HAM1													HAM1					
HAS1										HAS1								
HCA4										HCA4								
HEF3											HEF3							
HEM13								HEM13										
HEM14											HEM14	HEM14						
HEM15																Hem15		
HER1											HER1		HER1					
HEX3											HEX3							
HFA1											HFA1							
HFI1								HFI1			HFI1							
HFM1											HFM1							
HGH1								HGH1		HGH1	HGH1					Hgh1		
HHF1										HHF1								
HHF2										HHF2			HHF2					
HHO1												HHO1						
HIF1										HIF1			HIF1					
HIR2					HIR2					HIR2	HIR2							
HIR3					HIR3						HIR3		HIR3					
HIS5																His5		
HIS7					HIS7			HIS7			HIS7							
HLJ1											HLJ1							
HMT1								HMT1			HMT1							
HNT3					HNT3						HNT3							
HO											HO	HO						
HOF1						HOF1		HOF1		HOF1	HOF1		HOF1					
HOG1													HOG1			Hog1		
HOM3																Hom3		
HOS3					HOS3													
HPR1					HPR1			HPR1			HPR1	HPR1						
HRB1					HRB1	HRB1		HRB1		HRB1								
HRD3										HRD3	HRD3							
HSC82								HSC82		HSC82	HSC82					Hsc82	Hsc82	
HSH155										HSH155	HSH155							
HSH49											HSH49							
HSP104																Hsp104	Hsp104	
HSP12	HSP12																	
HSP42								HSP42		HSP42								
HSP60								HSP60										
HSP78										HSP78	HSP78					Hsp78		
HSP82										HSP82						Hsp82	Hsp82	
HUG1	Hug1																	
HUL4											HUL4							
HUL5											HUL5							
HXK1								HXK1										
HYM1								HYM1		HYM1	HYM1		HYM1					
HYP2										HYP2								
ICL1											ICL1							
ICL2							ICL2			ICL2								
ICT1								ICT1										
IDH1											IDH1					Idh1	Idh1	
IDH2					IDH2					IDH2						Idh2	Idh2	
IDI1													IDI1					
IDP1						IDP1					IDP1	IDP1				Idp1	Idp1	
IDP3								IDP3		IDP3	IDP3		IDP3					
IKI3								IKI3										
ILS1								ILS1										
ILV1																Ilv1		
ILV2																Ilv2	Ilv2	
ILV5								ILV5			ILV5							
ILV6								ILV6										

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*	TAP					All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
IMD1										IMD1								
IMD2												IMD2						
IMD3										IMD3						Imd3	Imd3	
IMD4										IMD4								
IMH1						IMH1		IMH1		IMH1	IMH1	IMH1						
IML1						IML1		IML1		IML1	IML1		IML1					
IML2								IML2			IML2					Iml2		
IMP3										IMP3								
INP53											INP53							
IOC3											IOC3							
IPP1	Ipp1																	
IQG1								IQG1			IQG1							
IRA2											IRA2							
IRE1					IRE1			IRE1		IRE1	IRE1		IRE1					
IRR1										IRR1								
ISA2										ISA2								
ISM1								ISM1										
ISR1	IRS1																	
ISW1								ISW1		IWS1	ISW1	ISW1						
ISW2					ISW2			ISW2		ISW2	ISW2							
ISY1											ISY1							
ITC1								ITC1			ITC1		ITC1					
JAC1											JAC1							
JJJ1											JJJ1							
JNM1										JNM1	JNM1							
JSN1																		
KAP123																Kap123	Kap123	
KAP95								KAP95								Kap95		
KAR3											KAR3							
KAR4													KAR4					
KCC4											KCC4							
KCS1					KCS1													
KEL1										KEL1	KEL1	KEL1	KEL1					
KEM1													KEM1					
KES1											KES1							
KGD1								KGD1			KGD1					Kgd1	Kgd1	
KGD2																Kgd2	Kgd2	
KIN1											KIN1							
KIN28													KIN28					
KIN4					KIN4						KIN4		KIN4					
KIP1											KIP1							
KIP2												KIP2						
KKQ8											KKQ8							
KNS1								KNS1		KNS1								
KOG1								KOG1		KOG1	KOG1							
KRE29											KRE29							
KRS1					KRS1	KRS1					KRS1							
KSS1								KSS1										
KTI12											KTI12							
KTR1											KTR1							
KTR2												KTR2						
KTR3													KTR3					
LAP4													LAP4					
LCB4								LCB4			LCB4							
LCB5											LCB5							
LCP5											LCP5		LCP5					
LEA1											LEA1							
LEU2																Leu2	Leu2	
LEU5						LEU5					LEU5							
LHP1							LHP1											
LHS1											LHS1		LHS1			Lhs1		
LIF1											LIF1							
LIG4											LIG4							
LIN1											LIN1							
LIP2								LIP2			LIP2							
LOT5					LOT5													
LPD1										LPD1						Lpd1	Lpd1	
LPE10											LPE10							
LRE1											LRE1							
LRG1												LRG1	LRG1					
LRS4											LRS4							
LSB3											LSB3							
LSB5										LSB5								
LSC1					LSC1			LSC1			LSC1	LSC1						
LSC2						LSC2												
LSG1									LSG1									
LSM1										LSM1								
LSM4										LSM4								
LSM5										LSM5								
LSM6																		
LSM7										LSM7								
LSP1	Lsp1																	
LST4											LST4							
LTE1								LTE1		LTE1	LTE1		LTE1					
LUC7											LUC7							
LUG1																		
LYS09											LYS9							
LYS1													LYS1			Lys1	Lys1	

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*		TAP				All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
LYS12																Lys12	Lys12	
LYS20																Lys20	Lys20	
LYS4																Lys4		
MAC1											MAC1							
MAD1											MAD1	MAD1						
MAD3										MAD3								
MAF1					MAF1													
MAK10						MAK10					MAK10							
MAK11											MAK11							
MAK21						MAK21												
MAK3								MAK3										
MAK5											MAK05		MAK05					
MAL32											MAL32	MAK32						
MAL33																		
MAM33								MAM33					MAM33					
MBF1	MBF1																	
MBP1											MBP1							
MCD1											MCD1							
MCM2								MCM2		MCM2	MCM2	MCM2						
MCM21								MCM21										
MCM3											MCM3							
MCM6								MCM6								Mcm6		
MCM7																Mcm7		
MCR1											MCR1					Mcr1		
MCT1											MCT1							
MDH1											MDH1							
MDH3																Mdh3		
MDJ1																	Mdj1	
MDL2											MDL2							
MDM1								MDM1			MDM01							
MDM12										MDM12								
MDM20												MDM20						
MDM38																Mdm38		
MDN1																Mdn1		
MDS3								MDS3			MDS3		MDS3					
MDV1					MDV1													
MED2											MED2							
MED4											MED4	MED4						
MEF1											MEF1		MEF1					
MEI4													MEI4					
MEK1									MEK1									
MES1											MES1	MES1						
MET1									MET1									
MET10										MET10						Met10	Met10	
MET13								MET13										
MET17										MET17						Met17	Met17	
MET18																Met18		
MET2													MET2					
MET3																Met3		
MET5																Met5		
MET6							MET6					MET06						
MFB1																		
MFT1								MFT1			MFT1							
MGA2											MGA2							
MGM1											MGM1							
MHP1					MHP1			MHP1		MHP1	MHP1		MHP1					
MHT1										MHT1								
MIP1													MIP1					
MIP6						MIP6				MIP6	MIP6							
MIR1																Mir1	Mir1	
MKT1													MKT1					
MLC1							MLC1					MLC1						
MLH2								MLH2			MLH2		MLH2					
MLP1					MLP1	MLP1		MLP1			MLP1	MLP1	MLP1					
MLP2								MLP2		MLP2	MLP2		MLP2					
MMS21									MMS21									
MMS22											MMS22							
MND2										MND2								
MNL1					MNL1													
MNN10																		
MNN4												MNN4						
MNN5											MNN5			MNN5				
MOD5					MOD5					MOD5			MOD5					
MOT1								MOT1		MOT1								
MPA43								MPA43										
MPC54											MPC54							
MPD1											MPD1							
MPD2						MPD2				MPD2	MPD2							
MPH1						MPH1					MPH1							
MPH3													MPH3					
MPM1																Mpm1		
MPP10											MPP10							
MPS2								MPS2										
MPT5											MPT5							
MRC1						MRC1												
MRE11								MRE11			MRE11							
MRF1								MRF1		MRF1	MRF1	MRF1	MRF1					
MRL1													MRL1					

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*	TAP					All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
MRP1											MRP1							
MRP13																Mrp13		
MRP2													MRP2					
MRP20											MRP20							
MRP4											MRP4							
MRP51											MRP51							
MRPL10										MRPL10	MRPL10	MRPL10	MRPL10					
MRPL15										MRPL15								
MRPL16										MRPL16	MRPL16							
MRPL19											MRPL19							
MRPL24					MRPL24													
MRPL3											MRPL3	MRPL3	MRPL3					
MRPL31											MRPL31							
MRPL35											MRPL35							
MRPL40								MRPL40		MRPL40	MRPL40	MRPL40	MRPL40					
MRPL49											MRPL49							
MRPL7								MRP7		MRP7	MRP7		MRP7					
MRPL9											MRPL09		MRPL09					
MRPS18					MRPS18						MRPS18							
MRPS28								MRPS28			MRPS28	MRPS28	MRPS28					
MRPS5								MRPS5		MRPS5	MRPS5							
MSB1							MSB1											
MSB4											MSB4		MSB4					
MSC1																		
MSC7											MSC7							
MSE1										MSE1	MSE1	MSE1	MSE1					
MSH1										MSH1	MSH1							
MSH3					MSH3			MSH3				MSH3						
MSH4											MSH4							
MSH6						MSH6		MSH6		MSH6	MSH6		MSH6					
MSI1											MSI1							
MSL5						MSL5					MSL5							
MSM1											MSM1							
MSN2													MSN2					
MSN5											MSN5		MSN5					
MSR1											MSR1							
MSS116											MSS116							
MSS2								MSS2		MSS2	MSS2	MSS2	MSS2					
MSS4											MSS4							
MSS51								MSS51										
MSY1								MSY1										
MTR4											MTR4							
MVP1								MVP1		MVP1	MVP1		MVP1			Mvp1		
MYO1								MYO1		MYO1	MYO1							
MYO2											MYO2					Myo2		
MYO4								MYO4			MYO4	MYO4						
NAR1							NAR1	NAR1										
NAS6											NAS6							
NAT1										NAT1	NAT1		NAT1					
NBP1											NBP1							
NCB2										NCB2								
NCE103											NCE103							
NCL1											NCL1	NCL1						
NCP1								NCP1										
NCR1											NCR1		NCR1					
NDE1											NDE1					Nde1	Nde1	
NDE2											NDE2							
NDI1																Ndi1	Ndi1	
NDT80										NDT80	NDT80							
NEO1											NEO1		NEO1					
NET1								NET1	NET1									
NEW1						NEW1				NEW1		NEW1						
NFI1													NFI1					
NFS1											NFS1		NFS1			Nfs1	Nfs1	
NGG1								NGG1										
NGL1											NGL1							
NIP1																Nip1		
NIP100					NIP100			NIP100		NIP100	NIP100		NIP100					
NIP7											NIP7							
NIT3											NIT3							
NMD2								NMD2		NMD2								
NMR1											NMR1		NMR1					
NOC4					NOC4						NOC4							
NOG2											NOG2							
NOP1										NOP1	NOP1					Nop1		
NOP14											NOP14							
NOP56																Nop56		
NOP58																Nop58	Nop58	
NOP8											NOP8							
NOT5											NOT5							
NPP1																		
NPR1													NPR1					
NPR2													NPR2					
NPT1												NPT1						
NRG1											NRG1							
NRG2						NRG2				NRG2	NRG2							
NSP1												NSP1						
NTG2													NTG2					

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*			TAP			All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
NTH2											NTH2							
NTO1						NTO1		NTO1			NTO1							
NUC1								NUC1										
NUP100										NUP100								
NUP133											NUP133		NUP133					
NUP157									NUP157		NUP157							
NUP159												NUP159	NUP159					
NUP170												NUP170	NUP170					
NUP82											NUP82							
NUP85											NUP85							
NUT1								NUT1										
NUT2											NUT2							
OCH1											OCH1							
OCT1					OCT1			OCT1			OCT1							
ODC1										ODC1	ODC1							
ODC2											ODC2							
OKP1						OKP1				OKP1								
OLA1																Ola1		
OLE1											OLE1							
OM45						OM45		OM45		OM45	OM45	OM45						
OPI1											OPI1							
ORC4											ORC4		ORC4					
OSH2											OSH2					Osh2		
OSH3												OSH3						
OSH7											OSH7							
OYE2	OYE2																	
PAB1																Pab1		
PAC2																		
PAD1											PAD1							
PAN2										PAN2	PAN2		PAN2					
PAN5										PAN5	PAN5							
PAP1											PAP1							
PAT1																		
PCA8																		
PCD1											PCD1							
PCI8 (YIL071C)					PCI8	PCI8	PCI8				PCI8							
PCL6										PCL6	PCL6		PCL6					
PCL7								PCL7			PCL7	PCL7						
PCL9											PCL9		PCL9					
PCT1											PCT1							
PDA1										PDA1						Pda1		
PDB1						PDB1										Pdb1		
PDC1																Pdc1		
PDH1								PDH1										
PDR10								PDR10										
PDR11											PDR11							
PDR15										PDR15								
PDR3											PDR03		PDR03					
PDS5								PDS5			PDS5		PDS5					
PDX1													PDX1					
PEP1											PEP1							
PEP3								PEP3		PEP3	PEP3		PEP3					
PEP4											PEP4							
PEP5										PEP5								
PER100					PER100													
PES4											PES4							
PET10																Pet10		
PET111										PET111	PET111		PET111					
PET123								PET123			PET123							
PET127											PET127							
PET130												PET130						
PET309											PET309							
PET56													PET56					
PET8					PET8									PET8				
PET9								PET9			PET9					Pet9	Pet9	
PEX11											PEX11							
PEX13											PEX13							
PEX14								PEX14			PEX14							
PEX15					PEX15													
PEX18							PEX18											
PEX19									PEX19			PEX19						
PEX2													PEX2					
PEX27					PEX27					PEX27	PEX27							
PEX3									PEX03									
PEX5								PEX5		PEX5	PEX5							
PEX8																		
PFD1					PFD1			PFD1			PFD1		PFD1					
PFK1											PFK1					Pfk1	Pfk1	
PFK2																Pfk2		
PFK26					PFK26			PFK26		PFK26	PFK26		PFK26					
PGD1													PGD1					
PGI1								PGI1										
PGK1																Pgk1		
PHA2													PHA2					
PHD1								PHD1		PHD1		PHD1						
PHO12													PHO12					
PHO13																		
PHO80													PHO80					

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*		TAP				All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
PHO85								PHO85					PHO85					
PHO86											PHO86							
PHO87																		
PHO90										PHO90								
PHR1											PHR1							
PIF1								PIF1		PIF1	PIF1							
PIG1						PIG1		PIG1										
PIL1													PIL1			Pil1		
PIM1					PIM1											Pim1		
PIP2									PIP2		PIP2							
PIR5																		
PKC1					PKC1			PKC1		PKC1	PKC1	PKC1	PKC1					
PKH1								PKH1			PKH1							
PKH2											PKH2							
PKR1																		
PLB3													PLB3					
PLC1											PLC1							
PLM2											PLM2		PLM2					
PMA1																Pma1	Pma1	
PMD1								PMD1			PMD1		PMD1					
PMI40													PMI40					
PMP1																		
PMT1											PMT1							
PMT2								PMT2			PMT2							
PNC1											PNC1		PNC1					
PNG1											PNG1							
POL1										POL01	POL1							
POL2								POL2		POL2	POL2							
POL30											POL30							
POL32								POL32					POL32					
POL4											POL4							
POM152								POM152			POM152							
POP2					POP2	POP2		POP2		POP2	POP2		POP2	POP2				
POR1							POR1									Por1	Por1	
POR2											POR2							
POT1										POT1	POT1							
POX1								POX1		POX1	POX1							
PPA2						PPA2												
PPG1								PPG1										
PPH21											PPH21							
PPH22											PPH22							
PPM1											PPM1							
PPN1													PPN1					
PPR1											PPR1							
PPS1											PPS1							
PPX1													PPX1					
PRB1											PRB1							
PRC1								PRC1										
PRD1											PRD1							
PRE10																Pre10		
PRE3											PRE3							
PRE6																Pre6		
PRE7											PRE7							
PRP19											PRP19		PRP19					
PRP2											PRP2	PRP2						
PRP22					PRP22	PRP22		PRP22		PRP22	PRP22		PRP22					
PRP3											PRP3							
PRP38													PRP38					
PRP45						PRP45		PRP45			PRP45							
PRP46									PRP46									
PRP5								PRP5		PRP5								
PRP8								PRP8		PRP8	PRP8		PRP8					
PRP9											PRP9							
PRR2							PRR2											
PRS1										PRS1								
PRT1								PRT1		PRT1	PRT1							
PSA1													PSA1			Psa1	Psa1	
PSE1																Pse1		
PSH1																		
PSL10								PSL10										
PSP1											PSP1							
PST1					PST1			PST1			PST1		PST1					
PST2																Pst2		
PTA1											PTA1							
PTC1											PTC1							
PTC2											PTC2							
PTC4											PTC4	PTC4						
PTC7											PTC7							
PTP1													PTP1					
PUF4								PUF4										
PUF6							PUF6											
PUP2								PUP2		PUP2				PUP2				
PUS1								PUS1			PUS1		PUS1					
PUS2								PUS2										
PUS6								PUS6										
PWP2								PWP2			PWP2		PWP2					
PXL1								PXL1										
PYC1											PYC1							

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*	TAP					All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	4 high score ***YIH1***	none **ATP11**	none ***CTL1***	6 high score **ILV1**	8 high score **ILV2**	5 high score **PAT1**	22 high score **Sem1**	4 high score **SHE3**	2 high score **SUI2**	none **VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
PYC2										PYC2	PYC2		PYC2					
PZF1											PZF1							
Q0050											Q0050		Q0050					
Q0060											Q0060	Q0060						
Q0065											Q0065							
Q0070										Q0070								
QNS1											QNS1							
QRC2																Qcr2	Qcr2	
QRI1													QRI1					
QRI2										QRI2								
QRI7											QRI7		QRI7					
QRI8					QRI8						QRI8							
RAD1					RAD1													
RAD10											RAD10		RAD10					
RAD2											RAD2		RAD2					
RAD3													RAD3					
RAD30											RAD30							
RAD4												RAD4	RAD4					
RAD5								RAD5			RAD5							
RAD50										RAD50	RAD50	RAD50						
RAD61											RAD61							
RAD9											RAD9							
RAS1										RAS1								
RAS2													RAS2					
RAT1													RAT1					
RAV1											RAV1		RAV1					
RAV2													RAV2					
RBG2	Rbg2															Rbg2		
RCK1											RCK1							
RCL1													RCL1					
RCY1											RCY1		RCY1					
RDS2					RDS2						RDS2		RDS2					
REC114											REC114							
REP1																Rep1		
RER2													RER2					
RET2																Ret2		
REX3								REX3		REX3	REX3		REX3					
RFA1											RFA1							
RFC2					RFC2					RFC2								
RFC3											RFC3	RFC3						
RFC4											RFC4	RFC4						
RFC5										RFC5								
RFX1											RFX1							
RGA2											RGA2							
RGD1								RGD1										
RGD2						RGD2												
RGR1											RGR1							
RGS2											RGS2							
RGT2								RGT2										
RHC18								RHC18			RHC18							
RHO4										RHO4								
RIB2													RIB2					
RIB3											RIB3							
RIB5										RIB5								
RIF1						RIF1					RIF1		RIF1	RIF1				
RIF2											RIF2							
RIM1													RIM1					
RIM15										RIM15	RIM15		RIM15					
RIM2						RIM2					RIM002							
RIM8													RIM8					
RIS1											RIS1		RIS1					
RLF2											RLF2							
RLI1											RLI1							
RLP7								RLP7										
RMA1											RMA1							
RMS1										RMS1								
RNA1																Rna1		
RNA14								RNA14		RNA14	RNA14							
RNH35											RNH35							
RNP1								RNP1		RNP1								
RNR1	RNR1															Rnr1	Rnr1	
RNR4												RNR4						
ROD1											ROD1		ROD1					
ROK1													ROK1					
ROM1											ROM1							
RPA135											RPA135							
RPA190													RPA190			Rpa190		
RPA49					RPA49											Rpa49		
RPB2																Rpb2		
RPC34						RPC34		RPC34		RPC34	RPC34							
RPC40																Rpc40		
RPD3												RPD3						
RPF2													RPF2					
RPG1									RPG1	RPG1	RPG1					Rpg1		
RPH1											RPH1							
RPL10								RPL10			RPL10					RPL10	RPL10	
RPL11A											RPL11A					RPL11A	RPL11A	
RPL12A																RPL12A	RPL12A	

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*			TAP			All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
RPL13A																RPL13A		
RPL13B																	RPL13B	
RPL14A																RPL14A		
RPL15A																RPL15A		
RPL15B								RPL15B		RPL15B	RPL15B		RPL15B					
RPL16A																RPL16A		
RPL16B																RPL16B		
RPL17A																RPL17A	RPL17A	
RPL18A											RPL18A		RPL18A			RPL18A		
RPL19A																RPL19A	RPL19A	
RPL1A																RPL1A	RPL1A	
RPL20A																RPL20A	RPL20A	
RPL20B																		
RPL21A																RPL21A	RPL21A	
RPL23A																RPL23A	RPL23A	
RPL24A																RPL24A	RPL24A	
RPL25																RPL25		
RPL26A																RPL26A		
RPL27A																RPL27A		
RPL28																RPL28	RPL28	
RPL2A																RPL2A	RPL2A	
RPL3																RPL3	RPL3	
RPL31A																RPL31A		
RPL33A																RPL33A		
RPL35A																	RPL35A	
RPL37A								RPL37A			RPL37A							
RPL40A								RPL40A		RPL40A	RPL40A							
RPL40B											RPL40B							
RPL43B								RPL43B										
RPL4A																RPL4A	RPL4A	
RPL4B																RPL4B	RPL4B	
RPL5																RPL5		
RPL6A																RPL6A		
RPL7A																RPL7A	RPL7A	
RPL8A																RPL8A	RPL8A	
RPL8B																RPL8B		
RPL9A																RPL9A		
RPN03											RPN3							
RPN09											RPN9							
RPN1											RPN1	RPN1						
RPN10											RPN10							
RPN11											RPN11							
RPN12											RPN12							
RPN13											RPN13							
RPN2											RPN2					Rpn2		
RPN7										RPN7	RPN7					Rpn7		
RPN8								RPN8		RPN8	RPN8							
RPO41								RPO41										
RPP0																RPP0	RPP0	
RPP1								RPP1		RPP1	RPP1	RPP1						
RPS0A																RPS0A	RPS0A	
RPS0B										RPS0B	RPS0B							
RPS10A													RPS10A					
RPS11A																RPS11A		
RPS13																RPS13		
RPS14A																RPS14A	RPS14A	
RPS16A																RPS16A		
RPS18A																RPS18A		
RPS1A																RPS1A		
RPS1B																RPS1B	RPS1B	
RPS2																RPS2	RPS2	
RPS20																RPS20		
RPS21B																		
RPS22A																RPS22A		
RPS22B											RPS22B							
RPS23A																RPS23A		
RPS24A																RPS24A		
RPS24B													RPS24B					
RPS26A																RPS26A		
RPS27B																		
RPS3																RPS3	RPS3	
RPS4A																RPS4A		
RPS5																RPS5		
RPS6A																RPS6A		
RPS7A																RPS7A		
RPS7B																RPS7B		
RPS8A																RPS8A		
RPS9A										RPS9A			RPS9A					
RPS9B								RPS9B		RPS9B			RPS9B			RPS9B		
RPT1											RPT1							
RPT2					RPT2								RPT2			Rpt2		
RPT3											RPT3		RPT3			Rpt3		
RPT4											RPT4					Rpt4		
RPT5								RPT5			RPT5					Rpt5		
RPT6								RPT6		RPT6	RPT6	RPT6	RPT6			RPT6		
RRB1											RRB1							
RRI1										RRI1								
RRM3								RRM3		RRM3	RRM3		RRM3					

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*			TAP			All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	4 high score *YIH1*	none **ATP11**	none *CTL1*	6 high score **ILV1**	8 high score **ILV2**	5 high score **PAT1**	22 high score **Sem1**	4 high score **SHE3**	2 high score **SUI2**	none **VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
RRN11											RRN11		RRN11					
RRN3											RRN3							
RRN5													RRN5					
RRN9						Rrn09				RRN9								
RRP12										RRP12	RRP12		RRP12					
RRP4											RRP4							
RRP43											RRP43		RRP43	RRP43				
RRP46											RRP46							
RRP5								RRP5		RRP5	RRP5		RRP5					
RRT12																		
RSC2											RSC2	RSC2						
RSC4								RSC4		RSC4	RSC4							
RSC58											RSC58							
RSC8											RSC8		RSC8					
RSE1											RSE1							
RSF1											RSF1							
RSM23											RSM23							
RSM24										RSM24								
RSM28										RSM28								
RTA1											RTA1							
RTN1																Rtn1		
RTN2											RTN2							
RTS2											RTS2							
RTT101										RTT101								
RTT103										RTT103								
RTT105											RTT105	RTT105						
RVB1																Rvb1	Rvb1	
RVB2																Rvb2	Rvb2	
RVS167								RVS167								Rvs167		
SAC3											SAC3							
SAC6																Sac6		
SAE3											SAE3							
SAM1					SAM1			SAM1		SAM1	SAM1					Sam1	Sam1	
SAM2								SAM2		SAM2	SAM2					Sam2		
SAP155												SAP155						
SAP185									SAP185									
SAR1																Sar1		
SAS3											SAS3							
SAS5													SAS5					
SAY1						SAY1		SAY1		SAY1	SAY1		SAY1					
SBE22											SBE22							
SCC2										SCC2								
SCD5											SCD5							
SCD6											SCD6		SCD6					
SCJ1											SCJ1							
SCM3											SCM3							
SCP160											SCP160							
SCS3											SCS3							
SCT1													SCT1					
SCW4											SCW4							
SDA1											SDA1							
SDH1																Sdh1		
SDL1									SDL1									
SDS23	Sds23									SDS23								
SDS24																Sds24		
SEC10											SEC10							
SEC18								SEC18			SEC18							
SEC2											SEC2							
SEC21																Sec21		
SEC26											SEC26					Sec26		
SEC27																Sec27		
SEC3											SEC3							
SEC4																Sec4		
SEC5											SEC5							
SEC53										SEC53								
SEC66								SEC63										
SEC7								SEC7			SEC7	SEC7				Sec7		
SEC8											SEC8							
SEH1											SEH1							
SEM1																		
SEN1								SEN1		SEN1	SEN1							
SEN54								SEN54										
SEO1													SEO1					
SER1						SER1		SER1								Ser1		
SET2											SET2							
SET3											SET3							
SET6					SET6			SET6			SET6							
SFA1											SFA1							
SFB2											SFB2		SFB2					
SFI1										SFI1	SFI1		SFI1					
SGA1											SGA1							
SGF73											SGF73							
SGS1					SGS1						SGS1							
SGT2											SGT2							
SHE1											SHE1							
SHE2												SHE2						
SHE3																		
SHE9								SHE9										

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*		TAP				All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	4 high score ***YIH1***	none **ATP11**	none ***CTL1***	6 high score **ILV1**	8 high score **ILV2**	5 high score **PAT1**	22 high score **Sem1**	4 high score **SHE3**	2 high score **SUI2**	none **VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
SHG1										SHG1		SHG1						
SHM1											SHM1							
SHM2										SHM2						Shm2	Shm2	
SHP1					SHP1	SHP1		SHP1		SHP1	SHP1	SHP1	SHP1					
SHR3											SHR3							
SHS1											SHS1							
SHY1											SHY1							
SIC1											SIC1							
SIK1								SIK1			SIK1		SIK1					
SIN3								SIN3										
SIN4								SIN4		SIN4								
SIP1											SIP1							
SIP3						SIP3					SIP3							
SIR4								SIR4	SIR4									
SIS1																Sis1		
SIW14										SIW14	SIW14	SIW14						
SIZ1										SIZ1	SIZ1							
SKI2										SKI2	SKI2							
SKI3								SKI3			SKI3	SKI3						
SKN1											SKN1							
SLA2							SLA2				SLA2					Sla2		
SLD3										SLD3								
SLH1								SLH1			SLH1		SLH1					
SLK19											SLK19							
SLN1							SLN1				SLN1							
SLU7										SLU7		SLU7						
SLX8											SLX8							
SLY1													SLY1					
SLZ1																		
SMB1											SMB1							
SMC1						SMC1		SMC1		SMC1	SMC1	SMC1	SMC1					
SMC2								SMC2		SMC2	SMC2	SMC2	SMC2					
SMC4										SMC4								
SMF1													SMF1					
SMK1								SMK1										
SMM1													SMM1					
SMY2							SMY2				SMY2							
SNF2											SNF2							
SNF3											SNF3							
SNF4																		
SNF5																		
SNF6											SNF6							
SNF8											SNF8	SNF8	SNF8					
SNQ2													SNQ2					
SNT1								SNT1		SNT1	SNT1		SNT1					
SNT2											SNT2	SNT2						
SNU114										SNU114	SNU114							
SNX4						SNX4					SNX4			SNX4				
SNZ2													SNZ2					
SOK1											SOK1							
SOL1											SOL1							
SOV1										SOV1	SOV1							
SPB1											SPB1							
SPB4											SPB4		SPB4					
SPC105											SPC105							
SPC3					SPC3									SPC3				
SPC42														SPC42				
SPC72									SPC72		SPC72							
SPC98							SPC98											
SPO14											SPO14							
SPO22					SPO22					SPO22	SPO22		SPO22					
SPO7											SPO7							
SPO71											SPO71							
SPO72						SPO72		SPO72			SPO72		SPO72					
SPO74											SPO74		SPO74					
SPO77											SPO77							
SPP1											SPP1							
SPR28					SPR28			SPR28				SPR28						
SPS18																		
SPT16													SPT16					
SPT20											SPT20							
SPT21										SPT21								
SPT3																		
SPT5										SPT5								
SPT6					SPT6						SPT06							
SRB2											SRB2		SRB2					
SRB4										SRB4	SRB4							
SRI1						SRI1		SRI1		SRI1	SRI1		SRI1					
SRL2											SRL2		SRL2					
SRN2								SRN2										
SRO7											SRO7							
SRP1											SRP1					Srp1		
SRP101											SRP101							
SRP40											SRP40							
SRP54											SRP54							
SRP72								SRP72			SRP72							
SRV2																Srv2		
SRY1											SRY1							

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*	TAP					All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	4 high score ***YIH1***	none **ATP11**	none ***CTL1***	6 high score **ILV1**	8 high score **ILV2**	5 high score **PAT1**	22 high score **Sem1**	4 high score **SHE3**	2 high score **SUI2**	none **VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
SSA1																Ssa1	Ssa1	
SSA2																Ssa2		
SSB1																	Ssb1	
SSB2									SSB2		SSB2					Ssb2		
SSC1					SSC1					SSC1						Ssc1	Ssc1	
SSD1											SSD1							
SSE2									SSE2		SSE2							
SSK1														SSK1				
SSK22						SSK22				SSK22	SSK22		SSK22	SSK22				
SSL1											SSL1							
SSN8											SSN8							
SSP134											SSP134							
SSQ1											SSQ1							
SST2											SST2	SST2						
SSZ1								SSZ1			SSZ1							
STB1					STB1	STB1		STB1										
STB6										STB6	STB6							
STD1									STD1									
STE11											STE11							
STE13											STE13							
STE2											STE2							
STE20					STE20						STE20							
STE23								STE23										
STE4								STE4		STE4								
STH1											STH1							
STM1								STM1								Stm1		
STP4									STP4									
STS1								STS1										
STT4											STT4							
STU1								STU1										
STU2								STU2			STU2							
SUB2																Sub2	Sub2	
SUC2											SUC2							
SUI1											SUI1							
SUI2																		
SUP45																Sup45		
SUR1						SUR1												
SUV3								SUV3			SUV3							
SWA2											SWA2							
SWD2								SWD2										
SWI3											SWI3							
SWI4										SWI4			SWI4					
SWR1								SWR1			SWR1							
SXM1																Sxm1		
SYF1										SYF1								
SYF2													SYF2					
SYG1											SYG1	SYG1	SYG1					
TAF30													TAF30					
TAL1											TAL1							
TAO3								TAO3			TAO3		TAO3					
TAT1											TAT1							
TBS1											TBS1							
TCM62								TCM62										
TCP1																Tcp1		
TDH1								TDH1		TDH1	TDH1					Tdh1		
TDH2					TDH2			TDH2		TDH2	TDH2					Tdh2		
TDH3																Tdh3	Tdh3	
TEA1					TEA1													
TEF1					TEF1	TEF1				TEF1	TEF1					Tef1	Tef1	
TEF2																		
TEF4										TEF4						Tef4		
TEL1								TEL1										
TEL2											TEL2							
TES1											TES1							
TFA2						TFA2				TFA2	TFA2							
TFB1											TFB1							
TFB3					TFB3						TFB3							
TFC1								TFC1			TFC1							
TFC3											TFC3							
TFC7								TFC7			TFC7	TFC7						
TFC8											TFC8							
TFP1								TFP1			TFP1							
TFS1											TFS1							
TGL2								TGL2			TGL2		TGL2					
TGL5	Tgl5																	
THI80										THI80	THI80							
THO1													THO1					
THP1																		
THR1										THR1								
THS1								THS1					THS1					
TID3						TID3		TID3			TID3		TID3					
TIF1										TIF1	TIF1					Tif1	Tif1	
TIF2										TIF2		TIF2						
TIF3						TIF3		TIF3		TIF3	TIF3							
TIF34																Tif34		
TIF35																Tif35		
TIF4632													TIF4632					
TIM44											TIM44		TIM44			Tim44		

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*		TAP				All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	4 high score ***YIH1***	none **ATP11**	none ***CTL1***	6 high score **ILV1**	8 high score **ILV2**	5 high score **PAT1**	22 high score **Sem1**	4 high score **SHE3**	2 high score **SUI2**	none **VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
TIP20										TIP20								
TIR1										TIR1								
TKL1								TKL1		TKL1								
TLG2										TLG2	TLG2							
TMA29							TMA29											
TOM20											TOM20							
TOM70												TOM70						
TOM71												TOM71						
TOP1						TOP1					TOP1		TOP1					
TOP2											TOP2							
TOP3											TOP3							
TOR1								TOR1		TOR1	TOR1		TOR1					
TOR2											TOR2		TOR2					
TPD3																Tpd3		
TPK1								TPK1										
TPK3										TPK3								
TPM1											TPM1							
TPO3													TPO3					
TPO4								TPO4										
TPS1											TPS1							
TPS2											TPS2							
TPS3											TPS3							
TRK1								TRK1			TRK1		TRK1					
TRK2										TRK2	TRK2		TRK2					
TRL1								TRL1		TRL1	TRL1	TRL1	TRL1					
TRM3											TRM3							
TRP2													TRP2					
TRP4											TRP4							
TRP5																Trp5	Trp5	
TRS120												TRS120						
TRS130								TRS130		TRS130	TRS130							
TSC13										TSC13	TSC13							
TSR3								TSR3					TSR3					
TUB1																Tub1		
TUB2								TUB2								Tub2	Tub2	
TUB3																Tub3		
TUF1											TUF1					Tuf1		
TWF1													TWF1					
TYS1								TYS1			TYS1							
TYW1								TYW1			TYW1							
UBA1							UBA1		UBA1		UBA1							
UBC1													UBC1					
UBC6																		
UBI4																Ubi4		
UBP1											UBP1							
UBP10								UBP10		UBP10								
UBP13						UBP13					UBP13							
UBP15							UBP15				UBP15							
UBP2											UBP2							
UBP5											UBP5							
UBP6											UBP6							
UBP9											UBP9							
UBR1																Ubr1		
UFD4										UFD4								
UFE1											UFE1							
UGA3											UGA3							
UGP1																Ugp1	Ugp1	
UGT51										UGT51	UGT51		UGT51					
UME6																		
UNG1											UNG1							
URA2					URA2	URA2		URA2		URA2	URA2			URA2		URA2	URA2	
URA3					URA3			URA3		URA3	URA3		URA3			Ura3	Ura3	
URA4											URA4							
URA5											URA5							
URA7																Ura7	Ura7	
URA8											URA8		URA8					
URE2								URE2										
URH1																		
USO1								USO1		USO1	USO1		USO1					
UTP10																Utp10		
UTP20								UTP20										
UTP6					UTP6							UTP6	UTP6					
VAC14											VAC14		VAC14					
VAM3						WHI2												
VAM6								VAM6			VAM6			VAM6				
VAM7						VAM7												
VAS1	Vas1							VAS1					VAS1					
VBA2																		
VID21								VID21			VID21		VID21					
VID24										VID24								
VID27											VID27		VID27					
VID28											VID28							
VID30						VID30												
VID31											VID31							
VIK1													VIK1					
VIP1						VIP1					VIP1					Vip1		
VMA1																Vma1		
VMA13																Vma13		

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*			TAP			All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	4 high score *YIH1*	none **ATP11**	none *CTL1*	6 high score **ILV1**	8 high score **ILV2**	5 high score **PAT1**	22 high score **Sem1**	4 high score **SHE3**	2 high score **SUI2**	none **VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
VMA2								VMA2			VMA2					Vma2	Vma2	
VMA5																Vma5	Vma5	
VMA8																Vma8		
VPH2								VPH2										
VPS1																Vps1	Vps1	
VPS13											VPS13							
VPS15											VPS15		VPS15					
VPS3								VPS3			VPS3							
VPS34													VPS34					
VPS35											VPS35							
VPS4										VPS4	VPS4							
VPS45											VPS45							
VTC3										VTC3	VTC3		VTC3					
VTH1											VTH1							
VTH2											VTH2							
WAR1					WAR1						WAR1							
WHI2						VID22												
WRS1										WRS1	WRS1							
XBP1							XBP1											
XCM1								XCM1			XCM1	XCM1						
XPT1													XPT1					
XRS2					XRS2						XRS2							
XYL2										XYL2								
YAL037W											YAL037W							
YAL045C											YAL045C							
YAL053W											YAL053W							
YAL061W					YAL061W													
YAL064CA													YAL064CA					
YAP0003											YAP0003							
YAP1											YAP1		YAP1					
YAP1801											YAP1801							
YAP1802													YAP1802					
YAP3						YAP3												
YAR010C							YAR010C				YAR010C							
YAR062W									YAR062W									
YAR064W											YAR064W	YAR064W						
YAR070C										YAR070C	YAR070C							
YAT2								YAT2			YAT2	YAT2						
YBL005WB											YBL005WB							
YBL009W											YBL009W							
YBL054W											YBL054W							
YBL055C											YBL055C		YBL055C					
YBL060W						YBL060W		YBL060W			YBL060W		YBL060W					
YBL098W									YBL098W									
YBL101WB											YBL101WB							
YBL111C											YBL111C							
YBR007C											YBR007C							
YBR012WB													YBR012WB					
YBR025C								YBR025C		YBR025C	YBR025C							
YBR089W													YBR089W					
YBR094W											YBR094W							
YBR168W										YBR168W	YBR168W							
YBR184W											YBR184W							
YBR203W											YBR203W							
YBR204C						YBR204C												
YBR246W								YBR246W										
YBR250W											YBR250W							
YBR255W										YBR255W								
YBR270C						YBR270C					YBR270C							
YBR284W													YBR284W					
YBR292C										YBR292C								
YBT1											YBT1							
YCF1											YCF1							
YCL001WB										YCL001WB								
YCL033C								YCL033C										
YCL039W								YCL039W										
YCL047C						YCL047C												
YCL048W											YCL048W							
YCL057CA											YCL057CA							
YCP4																Ycp4		
YCR022C								YCR022C										
YCR050C													YCR050C					
YCR076C											YCR076C							
YCR079W											YCR079W							
YCR095C								YCR095C			YCR095C							
YCR099C											YCR099C							
YCR105W								YCR105W		YCR105W	YCR105W	YCR105W						
YCS4													YCS4					
YDJ1																Ydj1	Ydj1	
YDL027C								YDL027C		YDL027C	YDL027C		YDL027C					
YDL033C											YDL033C							
YDL062W											YDL062W							
YDL063C												YDL063C						
YDL068W											YDL068W							
YDL094C							YDL094C											
YDL100C								YDL100C										
YDL109C											YDL109C							
YDL119C						YDL119C												

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*		TAP				All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
YDL133W											YDL133W							
YDL146W								YDL146W			YDL146W							
YDL156W										YDL156W								
YDL176W											YDL176W							
YDL199C											YDL199C							
YDL206W											YDL206W							
YDL221W										YDL221W	YDL221W							
YDL223C								YDL223C										
YDL246C								YDL246C										
YDR015C											YDR015C							
YDR034CD										YDR034CD	YDR034CD							
YDR042C											YDR042C							
YDR051C										YDR051C				YDR051C				
YDR053W									YDR053W									
YDR095C													YDR095C					
YDR098CB											YDR098CB							
YDR101C								YDR101C		YDR101C								
YDR117C													YDR117C					
YDR132C											YDR132C							
YDR165W												YDR165W						
YDR185C											YDR185C							
YDR186C								YDR186C			YDR186C							
YDR187C										YDR187C								
YDR222W								YDR222W										
YDR233C											YDR233C							
YDR239C											YDR239C							
YDR249C										YDR249C	YDR249C							
YDR261CD											YDR261CD	YDR261CD						
YDR275W											YDR275W							
YDR282C								YDR282C										
YDR291W								YDR291W				YDR291W						
YDR306C						YDR306C		YDR306C		YDR306C	YDR306C	YDR306C	YDR306C					
YDR314C											YDR314C							
YDR316WB											YDR316WB							
YDR324C								YDR324C		YDR324C	YDR324C							
YDR326C													YDR326C					
YDR330W											YDR330W							
YDR332W						YDR332W		YDR332W										
YDR333C											YDR333C							
YDR341C											YDR341C		YDR341C					
YDR348C											YDR348C							
YDR365C											YDR365C							
YDR365WB											YDR365WB							
YDR372C										YDR372C	YDR372C							
YDR391C											YDR391C							
YDR442W										YDR442W								
YDR444W								YDR444W										
YDR458C											YDR458C							
YDR459C								YDR459C										
YDR466W					YDR466W	YDR466W				YDR466W	YDR466W		YDR466W					
YDR474C											YDR474C							
YDR475C													YDR475C					
YDR485C											YDR485C							
YDR514C								YDR514C				YDR514C						
YDR520C											YDR520C							
YDR541C								YDR541C			YDR541C							
YEF3								YEF3								Yef3	Yef3	
YEL010W											YEL010W							
YEL017W										YEL017W	YEL017W							
YEL023C							YEL023C											
YEL076C								YEL076C										
YEL077C					YEL077C													
YEN1						YEN1		YEN1										
YER004W											YER004W		YER004W					
YER053C												YER053C						
YER064C													YER064C					
YER066W					YER066W						YER066W							
YER077C										YER077C								
YER080W								YER080W			YER080W							
YER097W											YER097W		YER080W					
YER140W					YER140W			YER140W		YER140W	YER140W	YER140W	YER140W					
YER156C								YER156C		YER156C								
YER160C										YER160C								
YER182W											YER182W							
YER184C											YER184C							
YER185W								YER185W										
YFL042C										YFL042C								
YFL044C											YFL044C							
YFL049W													YFL049W					
YFL054C											YFL054C	YFL054C	YFL054C					
YFL067W								YFL067W										
YFR006W											YFR006W							
YFR016C											YFR016C		YFR016C					
YFR024C							YFR024C	YFR024C										
YFR041C											YFR041C							
YFR044C								YFR044C			YFR044C							
YGL050W											YGL050W							
YGL060W													YGL060W					

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*		TAP				All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
YGL082W													YGL082W					
YGL098W					YGL098W						YGL098W							
YGL139W													YGL139W					
YGL140C					YGL140C													
YGL245W								YGL245W										
YGR002C								YGR002C			YGR002C							
YGR012W													YGR012W					
YGR027WB											YGR027WB							
YGR046W								YGR046W		YGR046W	YGR046W							
YGR052W								YGR052W			YGR052W							
YGR054W					YGR054W					YGR054W								
YGR058W											YGR058W							
YGR064W											YGR064W							
YGR066C								YGR066C			YGR066C							
YGR067C								YGR067C										
YGR069W											YGR069W							
YGR090W											YGR090W		YGR090W					
YGR096W											YGR096W							
YGR109WB											YGR109WB							
YGR122W											YGR122W							
YGR126W								YGR126W										
YGR130C						YGR130C		YGR130C		YGR130C	YGR130C							
YGR149W								YGR149W										
YGR154C					YGR154C			YGR154C			YGR154C		YGR154C					
YGR161C					YGR161C					YGR161C	YGR161C		YGR161C					
YGR161CD											YGR161CD							
YGR165W													YGR165W					
YGR168C											YGR168C							
YGR173W											YGR173W							
YGR203W								YGR203W										
YGR235C											YGR235C							
YGR237C								YGR237C										
YGR265W											YGR265W							
YGR266W											YGR266W							
YGR287C											YGR287C							
YHB1																Yhb1	Yhb1	
YHC1											YHC1							
YHL009WB											YHL009WB		YHL009WB					
YHL035C													YHL035C					
YHM2																	Yhm2	
YHR003C											YHR003C							
YHR020W													YHR020W					
YHR033W								YHR033W		YHR033W						YHR033W		
YHR035W											YHR035W							
YHR040W										YHR040W	YHR040W							
YHR049W											YHR049W							
YHR080C					YHR080C						YHR080C							
YHR097C								YHR097C			YHR097C							
YHR112C											YHR112C							
YHR126C											YHR126C		YHR126C					
YHR132WA										YHR132WA		YHR132WA						
YHR149C										YHR149C								
YHR155W					YHR155W								YHR155W					
YHR176W								YHR176W			YHR176W	YHR176W						
YHR197W							YHR197W											
YHR199C											YHR199C							
YIL019W											YIL019W							
YIL082WA											YIL082WA							
YIL083C													YIL083C					
YIL091C										YIL091C								
YIL092W										YIL092W			YIL092W					
YIL112W										YIL112W	YIL112W	YIL112W	YIL112W					
YIL113W								YIL113W		YIL113W								
YIL121W													YIL121W					
YIL157C											YIL157C							
YIL158W								YIL158W				YIL158W						
YIL177C								YIL177C				YIL177C						
YIR003W											YIR003W		YIR003W					
YIR010W													YIR010W					
YIR014W											YIR014W							
YIR016W													YIR016W					
YIR035C							YIR035C											
YIR042C											YIR042C							
YJL037W					YJL037W			YJL037W										
YJL051W											YJL051W							
YJL083W					YJL083W													
YJL084C								YJL084C			YJL084C	YJL084C						
YJL103C							YJL103C			YJL103C	YJL103C							
YJL107C		YJL107C						YJL107C		YJR107W								
YJL109C											YJL109C							
YJL113W										YJL113W								
YJL131C								YJL131C		YJL131C	YJL131C		YJL131C					
YJL132W											YJL132W							
YJL145W													YJL145W					
YJL149W						YJL149W							YJL171C					
YJL185C								YJL185C			YJL185C							
YJL192C											YJL192C							
YJL200C								YJL200C			YJL200C							

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*	TAP									*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	4 high score ***YIH1***	none **ATP11**	none ***CTL1***	6 high score **ILV1**	8 high score **ILV2**	5 high score **PAT1**	22 high score **Sem1**	4 high score **SHE3**	2 high score **SUI2**	none **VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
YJL206C					YJL206C													
YJL207C								YJL207C			YJL207C							
YJL211C								YJR011C										
YJL225C											YJL225C	YJL225C						
YJR011C											YJR011C							
YJR030C											YJR030C							
YJR039W											YJR039W							
YJR072C											YJR072C							
YJR080C											YJR080C							
YJR083C											YJR083C							
YJR096W								YJR096W										
YJR100C													YJR100C					
YJR107W					YJR107W	YJR107W		YJR107W			YJR107W		YJR107W					
YJR119C											YJR119C							
YJR124C											YJR124C							
YJR126C								YJR126C					YJR126C					
YJR129C						YJR129C							YJR129C					
YJR136C								YJR136C										
YJR141W								YJR141W										
YJR154W											YJR154W							
YJU2					YJU2			YJU2			YJU2	YJU2	YJU2					
YJU3										YJU3								
YKL014C											YKL014C		YKL014C					
YKL077W											YKL077W							
YKL091C											YKL091C							
YKL098W					YKL098W									YKL098W				
YKL100C											YKL100C							
YKL105C											YKL105C		YKL105C	YKL105C				
YKL107W						YKL107W												
YKL121W										YKL121W								
YKL137W											YKL137W							
YKL147C											YKL147C							
YKL151C							YKL151C											
YKL160W											YKL160W							
YKL171W											YKL171W		YKL171W					
YKL195W								YKL195W			YKL195W	YKL195W	YKL195W					
YKL214C											YKL214C							
YKL215C					YKL215C			YKL215C		YKL215C	YKL215C							
YKR015C											YKR015C							
YKR016W											YKR016W							
YKR017C											YKR017C							
YKR018C																YKR018C		
YKR023W											YKR023W	YKR023W						
YKR038C											YKR038C							
YKR064W										YKR064W								
YKR078W											YKR078W							
YKR079C											YKR079C							
YKR096W													YKR096W					
YKR103W											YKR103W							
YKR104W											YKR104W							
YKT6																Ykt6		
YLL023C								YLL023C										
YLL032C											YLL032C							
YLL033W								YLL033W			YLL033W							
YLL034C								YLL034C										
YLL054C											YLL054C							
YLL058W													YLL058W					
YLR001C											YLR001C							
YLR016C											YLR016C							
YLR021W													YLR021W					
YLR023C										YLR023C	YLR023C							
YLR035CA										YLR035CA								
YLR046C											YLR046C							
YLR047C											YLR047C							
YLR049C											YLR049C		YLR049C					
YLR051C													YLR051C					
YLR054C													YLR054C					
YLR063W					YLR063W					YLR063W								
YLR073C											YLR073C							
YLR097C								YLR097C										
YLR108C											YLR108C		YLR108C					
YLR112W								YLR112W										
YLR122C											YLR122C							
YLR173W					YLR173W													
YLR187W								YLR187W			YLR187W							
YLR193C													YLR193C					
YLR199C											YLR199C							
YLR224W											YLR224W		YLR224W					
YLR225C											YLR225C							
YLR230W								YLR230W										
YLR247C													YLR247C					
YLR251W																		
YLR253W													YLR253W					
YLR255C								YLR255C										
YLR271W													YLR271W					
YLR278C											YLR278C							
YLR283W											YLR283W							
YLR290C										YLR290C	YLR290C							

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*			TAP			All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
YLR312C								YLR312C		YLR312C	YLR312C		YLR312C					
YLR352W											YLR352W							
YLR381W											YLR381W							
YLR387C										YLR387C								
YLR404W											YLR404W		YLR404W					
YLR405W											YLR405W							
YLR409W											YLR409C							
YLR419W											YLR419W	YLR419W						
YLR422W								YLR422W			YLR422W		YLR422W					
YLR424W											YLR424W	YLR424W						
YLR427W											YLR427W							
YLR431C											YLR431C							
YLR446W											YLR446W							
YLR454W									YLR454W									
YLR455W								YLR455W										
YML013W											YML013W							
YML014W												YML014W						
YML059C											YML059C							
YML072C								YML072C			YML072C		YML072C					
YML081W											YML081W							
YML082W											YML082W							
YML083C											YML083C							
YML089C								YML089C										
YML093W					YML093W													
YML107C								YML107C				YML107C						
YML117W									YML117W		YML117W							
YMR031W-A								YMR031C		YMR031C	YMR031C							
YMR034C								YMR034C										
YMR041C													YMR041C					
YMR045C											YMR045C							
YMR068W											YMR068W							
YMR073C													YMR073C					
YMR085W											YMR085W							
YMR086W										YMR086W								
YMR090W											YMR090W							
YMR097C								YMR097C		YMR097C		YMR097C						
YMR098C											YMR098C							
YMR099C								YMR099C										
YMR102C								YMR102C										
YMR114C					YMR114C			YMR114C			YMR114C							
YMR130W											YMR130W							
YMR160W									YMR160W									
YMR163C										YMR163C								
YMR184W								YMR184W										
YMR191W											YMR191W							
YMR196W								YMR196W										
YMR206W							YMR206W											
YMR226C											YMR226C							
YMR247C								YMR247C										
YMR251W												YMR251W						
YMR259C								YMR259C		YMR259C		YMR259C	YMR259C					
YMR262W								YMR262W			YMR262W		YMR262W					
YMR265C										YMR265C	YMR265C							
YMR289W								YMR289W			YMR289W							
YMR291W							YMR291W											
YMR306CA											YMR306CA							
YNG1											YNG1		YNG1					
YNL011C											YNL011C							
YNL040W								YNL040W		YNL040W								
YNL047C											YNL047C							
YNL063W											YNL063W							
YNL087W								YNL087W		YNL087W	YNL087W							
YNL089C								YNL089C			YNL089C		YNL089C					
YNL108C								YNL108C		YNL108C								
YNL120C													YNL120C					
YNL129W											YNL129W							
YNL143C											YNL143C							
YNL144C								YNL144C										
YNL177C											YNL177C							
YNL195C					YNL195C			YNL195C		YNL195C	YNL195C		YNL195C					
YNL234W								YNL234W		YNL234W	YNL234W	YNL234W	YNL234W					
YNL275W								YNL275W										
YNL284CB											YNL284CB	YNL284CB	YNL284CB					
YNL305C													YNL305C					
YNP5																		
YNR021W								YNR021W										
YNR029C								YNR029C			YNR029C		YNR029C					
YNR034WA													YNR034WA					
YNR040W						YNR040W												
YNR062C										YNR062C								
YNR063W											YNR063W							
YNR070W											YNR070W							
YOL034W								YOL034W					YOL034W					
YOL037C								YOL037C										
YOL053W								YOL053W										
YOL054W								YOL054W			YOL054W							
YOL075C													YOL075C					
YOL098C								YOL098C		YOL098C	YOL098C		YOL098C					

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006* TAP						All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
YOL101C											YOL101C							
YOL103WA											YOL103WA							
YOL103WB											YOL103WB							
YOL111C										YOL111C								
YOL117W								YOL117W			YOL117W							
YOL128C										YOL128C								
YOL137W													YOL137W					
YOL150C								YOL150C										
YOL164W											YOL164W							
YOP1																Yop1		
YOR004W											YOR004W		YOR004W					
YOR019W											YOR019W							
YOR021C											YOR021C							
YOR041C											YOR041C							
YOR042W													YOR042W					
YOR059C								YOR059C										
YOR081C								YOR081C										
YOR086C													YOR086C					
YOR093C								YOR093C		YOR093C	YOR093C							
YOR1						YOR001		YOR001		YOR001	YOR001							
YOR111W											YOR111W							
YOR112W								YOR112W			YOR112W	YOR112W	YOR112W					
YOR142WB											YOR142WB							
YOR155C											YOR155C							
YOR192CB								YOR192CB										
YOR205C								YOR205C										
YOR215C					YOR215C													
YOR220W											YOR220W							
YOR223W													YOR223W					
YOR225W											YOR225W							
YOR228C											YOR228C							
YOR243C												YOR243C						
YOR251C											YOR251C		YOR251C					
YOR255W								YOR255W		YOR255W	YOR255W							
YOR262W											YOR262W							
YOR271C						YOR271C		YOR271C										
YOR280C											YOR280C							
YOR296W								YOR296W		YOR296W			YOR296W					
YOR305W												YOR305W						
YOR315W						YOR315W												
YOR320C										YOR320C								
YOR343C										YOR343C	YOR343C		YOR343C					
YOR379C											YOR379C							
YOR396W													YOR396W					
YOX1											YOX1							
YPK1																Ypk1		
YPL009C										YPL009C								
YPL017C						YPL017C												
YPL030W											YPL030W							
YPL039W											YPL039W							
YPL070W											YPL070W							
YPL073C											YPL073C							
YPL088W											YPL088W		YPL088W					
YPL109C								YPL109C										
YPL137C								YPL137C		YPL137C	YPL137C	YPL137C						
YPL141C								YPL150W										
YPL159C					YPL159C			YPL159C		YPL159C	YPL159C							
YPL183C											YPL183C		YPL183C					
YPL216W											YPL216W							
YPL222W											YPL222W		YPL222W					
YPL225W											YPL225W							
YPL230W											YPL230W							
YPL247C									YPL247C									
YPL260W											YPL260W							
YPL267W					YPL267W													
YPR004C										YPR004C								
YPR022C								YPR022C										
YPR039W											YPR039W							
YPR064W											YPR064W							
YPR078C					YPR078C	YPR078C		YPR078C			YPR078C	YPR078C	YPR078C					
YPR083W										YPR083W								
YPR097W											YPR097W							
YPR112C											YPR112C							
YPR115W						YPR115W		YPR115W		YPR115W			YPR115W					
YPR117W											YPR117W							
YPR140W								YPR140W		YPR140W	YPR140W		YPR140W	YPR140W				
YPR148C													YPR148C					
YPR158W											YPR158W							
YPR172W													YPR172W					
YPR204W										YPR204W								
YPT1								YPT1			YPT1	YPT1				Ypt1		
YPT11											YPT11							
YPT32											YPT32							
YPT53					YPT53			YPT53			YPT53							
YRA1					YRA1			YRA1		YRA1	YRA1	YRA1	YRA1					
YRB2									YRB2	YRB2	YRB2							
YRF11										YRF11			YRF11					
YRF12										YRF12	YRF12		YRF12					

	Tarassov 2008	*Ito 2001*	*Uetz 2000*		*Krogan 2006*	TAP					All baits are low score (<0.02)				*Gavin 2006*	*In-house*		
	PCA	Y2H	Y2H		includes low-scored interactions, high score are underlined						italics: also as YBP-BP				TAP			
					4 high score	none	none	6 high score	8 high score	5 high score	22 high score	4 high score	2 high score	none				
prey \ bait ->	**GCN1**	**JSN1**	**URH1**	**Pir5**	***YIH1***	**ATP11**	***CTL1***	**ILV1**	**ILV2**	**PAT1**	**Sem1**	**SHE3**	**SUI2**	**VPS1**	**YIH1**	**YIH1*H2**	**YIH1**	**GST**
YRF14													YRF14					
YRF16								YRF16										
YSC83					YSC83						YSC83	YSC83						
YTA6								YTA6			YTA6							
ZDS1											ZDS1		ZDS1					
ZDS2											ZDS2							
ZIP1										ZIP1			ZIP1					
ZIP2							ZIP2				ZIP2							
ZMS1								ZMS1		ZMS1	ZMS1							
ZPR1																Zpr1		
ZRT1								ZRT1			ZRT1							
ZTA1												ZTA1						
ZWF1											ZWF1					Zwf1		

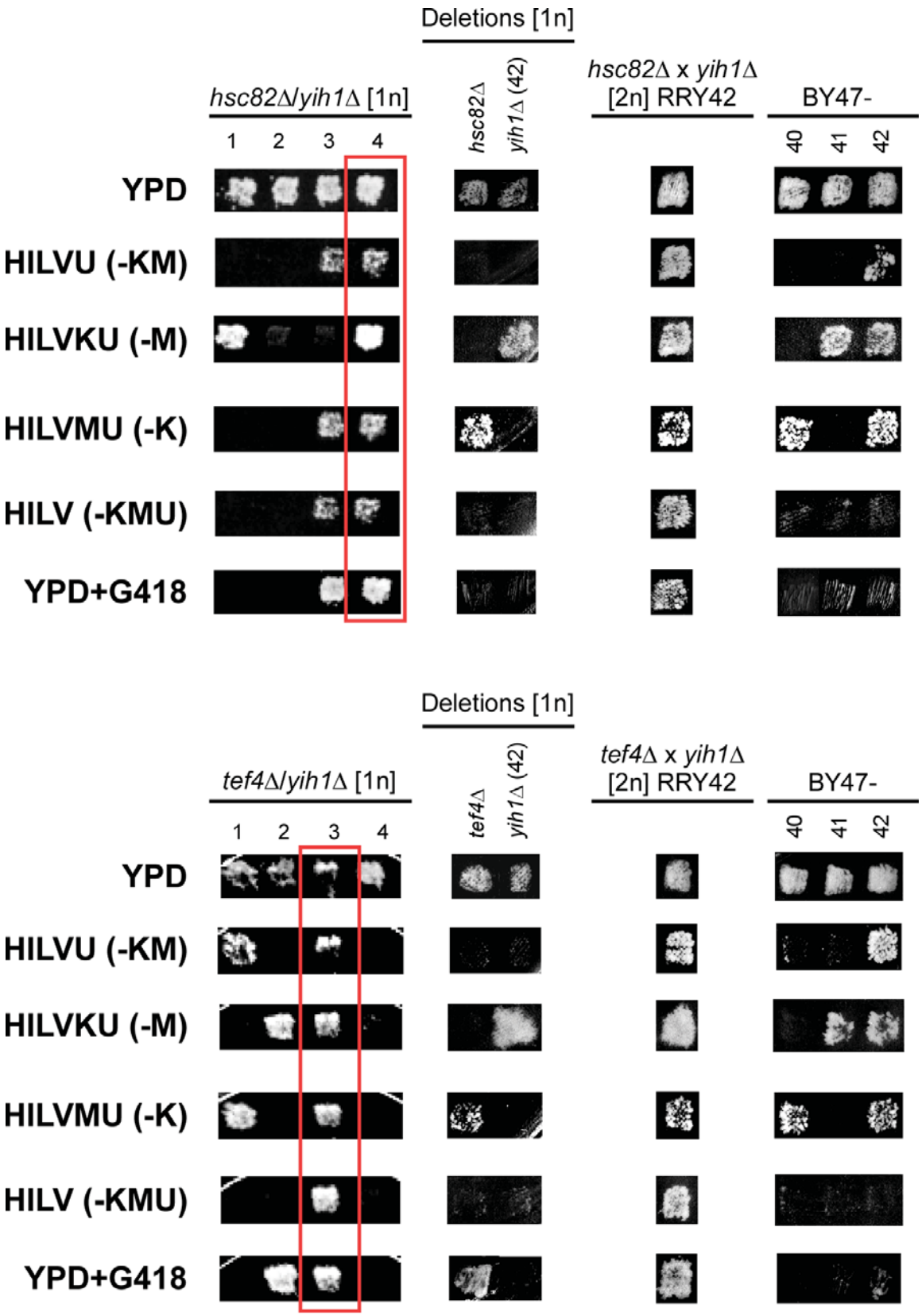
Appendix B: List of proteins that were tested for SM sensitivity by Parsons et al. (2004) and that were found as published putative and in-house putative YBP

ADH1	CYS4	KAR3	PGD1	SAC3	SRO7	VMA8
ADK1	DBF2	KTI12	PHO80	SAC6	SRV2	VPH2
ANP1	DDC1	LCB4	PLC1	SAP155	SSD1	VPS1
APE2	DEG1	LEA1	PMT2	SCP160	SSK1	VPS15
APG7	DEP1	LSM1	POL32	SEC66	SSZ1	VPS3
ARD1	DIA2	LST4	POP2	SEM1	SWR1	VPS35
ARO1	DNF1	MAD1	PSL10	SEO1	TEF4	VPS4
ARO2	DOA4	MAK10	PTC1	SER1	THP1	XRS2
ARV1	DUN1	MCT1	PUF6	SET3	THR1	YBR094W
ATP5	EAP1	MDM20	RAD4	SET6	TIF1	YCR079W
BCK1	ECM8	MET18	RAD5	SGF73	TIF3	YDJ1
BEM1	ERG5	MFT1	RAD50	SGS1	TLG2	YDR442W
BEM2	ERG6	MMS22	RAD9	SHE9	TOP1	YFR041C
BFR1	FAB1	MNN10	RAV1	SHM1	TOP3	YGL050W
BIK1	FKS1	MRC1	RCY1	SHP1	TOR1	YGR161C
BMH1	GAL11	MRE11	RGD1	SHS1	TPS1	YIL092W
BMH2	GAS1	MRPL31	RIM15	SHY1	TUB3	YLL023C
BRE1	GCN1	MRPL7	RPA49	SIN3	UBP15	YLR047C
BRO1	GCN2	NCL1	RPL13B	SIN4	UBP2	YML117W
BUB1	GCN20	NDE1	RPL14A	SIW14	UBP6	YMR031W-A
BUD22	GCN3	NGG1	RPL19A	SLX8	UME6	YMR073C
CCE1	GCV2	NIP100	RPL20B	SNF2	URA5	YMR191W
CCZ1	GRR1	NUP133	RPL21A	SNF5	URE2	YOR320C
CDC73	HIR2	NUT1	RPL27A	SNF6	VAC14	YOR343C
CDH1	HOF1	OCT1	RPL35A	SNF8	VAM3	YPK1
CHL1	HOM3	OPI1	RPL8B	SNT1	VAM6	YPT11
CIN8	HSC82	PAC2	RPS10A	SNX4	VAM7	
CLA4	ILV6	PAT1	RPS16A	SPC72	VID21	
CLC1	INP53	PET309	RPS22A	SPT20	VID31	
CSR1	IRE1	PET56	RSC2	SPT3	VMA13	
CTF04	ISW1	PFD1	RTT101	SRB2	VMA2	
CVT9	JNM1	PFK2	RVS167	SRN2	VMA5	

Appendix C: Verification of the double deletion strains constructed in Chapter E.2 and E.3

Shown are the results of representative dot tests for the double deletions and controls on rich (YPD) and different selection media. Each row shows the result of a single petri dish. “1n” and “2n” refer to haploids and diploids, respectively. “*yih1*Δ (42)” refers to the *YIH1* deletion strain RRY42. The diploid strain (third column) was created by mating the indicated haploid deletion strains and was used for the tetrad dissection. The four colonies in the first column were derived from the four cells of one tetrad. Amino acids that are not present in the medium (based on the selectable markers of the haploid deletion strains, cf. Table B3) are given in parenthesis.

The strain highlighted with the red rectangle was selected for the dot test in Chapters E.2 and E.3 (MBY77-2-11a and MBY77-3-4a in Table B3). These cells were growing on all selection media, indicating a successful complementation of the individual auxotrophies and the *KanMX* resistance marker and therefore indicating a double deletion. As Hsc82 and Hsp82 are 97% similar in their amino acids sequence (Borkovich et al., 1989) an attempt was made to construct *hsp82*Δ/*yih1*Δ double deletion strains; however, their verification was not successful.


Deletions [1n]
hsc82Δ/yih1Δ [1n]
1 2 3 4
hsc82Δ
yih1Δ (42)
hsc82Δ x yih1Δ
[2n] RRY42
BY47-
40 41 42
YPD
HILVU (-KM)
HILVKU (-M)
HILVMU (-K)
HILV (-KMU)
YPD+G418
Deletions [1n]
tef4Δ/yih1Δ [1n]
1 2 3 4
tef4Δ
yih1Δ (42)
tef4Δ x yih1Δ
[2n] RRY42
BY47-
40 41 42
YPD
HILVU (-KM)
HILVKU (-M)
HILVMU (-K)
HILV (-KMU)
YPD+G418

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