Large scale investigation to identify novel gene(s)
involved in translation pathway in *Saccharomyces
cerevisiae*.

by

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ABSTRACT

Translation initiation is one of the key regulatory steps in the protein biosynthesis pathway. Prior research identified numerous variations in this mechanism optimizing cellular performance during specific conditions. Of particular interest is an alternative mode of translation initiation via an internal ribosomal entry site (IRES), that is associated with stress response. A multitude of IRES-specific trans-acting factors (ITAFs) have been identified to date. In this investigation, we hypothesized that many ITAFs remain undiscovered. We conducted a large-scale screen in *S. cerevisiae* using a *LacZ* reporter under IRES control to identify genes positively regulating IRES-mediated initiation. Six genes were selected as potential ITAFs, and their genetic interaction networks were explored for the links to the translation process. We found associations between our candidates and the genes involved in both translation and stress response mechanisms, supporting their proposed function as ITAFs and demonstrating the potential for further investigations to identify regulators of IRES-mediated translation.

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LIST OF ACRONYMS

β -galactosidase	beta- galactosidase
Δ	deletion
$^{\circ}\text{C}$	degrees Celsius
μL	microliter
3-AT	3-aminotriazole
4E-BP1	eIF4E-binding protein 1
A.U.	activity units
AmpR	ampicillin resistance
Arg	arginine
ATP	adenosine triphosphate
Cana	canavanine
CPY	carboxypeptidase Y
DMSO	dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
eRF	eukaryotic release factor
GDP	guanosine diphosphate
GO	gene ontology
GTP	guanosine triphosphate
His	histidine
hnRNP	heterogeneous ribonucleoprotein
IRES	internal ribosomal entry site
ITAF	IRES trans-acting factor
Kan	kanamycin
LiAc	lithium acetate
Lys	lysine
MATa	mating type a
MAT α	mating type α
mg	milligram
mL	milliliter
mM	millimolar
mRNA	messenger RNA

MS	mass spectrometry
MSG	monosodium glutamate
mTOR	mechanistic target of rapamycin
NatR	nourseothricin resistance
ng	nanogram
NPC	nucleopore complex
OD	optical density
ONPG	ortho-Nitrophenyl- β -galactoside
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
PSA	phenotypic suppression analysis
PTB	polypyrimidine-tract-binding protein
RNP	ribonucleoprotein
RQC	ribosome quality control
rRNA	ribosomal RNA
SDL	synthetic dosage lethality
SGA	synthetic genetic array
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
TAE	Tris-acetate-EDTA (buffer)
TAP	tandem affinity purification
TC	ternary complex
Thia	thialysine
THSC	<i>Thp1-Sac3-Sus1-Cdc31</i> complex
TOR	target of rapamycin
TORC1/2	target of rapamycin complex 1/2
TRAMP	<i>Trf4p/Air2p/Mtr4p</i> polyadenylation complex
tRNA	transfer RNA
uORF	upstream open reading frame
UTR	untranslated region
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
YPD	yeast extract-peptone-dextrose (media)

CHAPTER 1. INTRODUCTION

In a broader biological context, translation often refers to a series of complex steps and processes that together lead to the synthesis of new proteins. The mechanism involves scanning information encoded by the mRNA and translating it into a chain of amino acids. The process of translation is mainly conducted by means of the large and small ribosomal subunits and associated proteins. Eukaryotes possess 40S small subunit consisting of 18S ribosomal RNA (rRNA) and 33 associated proteins, as well as 60S large ribosomal subunit, consisting of 5S, 5.8S and 28S rRNAs with 46 associated proteins.¹ Traditionally, translation has been subdivided into initiation, elongation, and termination; although recently it was proposed to place charging tRNAs with their corresponding amino acids as the first step of translation.²

Translation initiation is the focus of this research. It is an introductory, rate limiting step in the translation process when the machinery is being assembled and activated. Therefore, it is strictly and carefully regulated against malfunction. For the same reasons, it exhibits certain diversity in its mechanism of action in accordance with the cellular requirements.

1.1 Canonical Model of Eukaryotic Translation

Canonical translation initiation is highly dependent on translational machinery recognizing and scanning the 7-methylguanosine (m7G) cap structure.³ Initially, eIF4F heterodimer protein complex binds the cap. It consists of eIF4A (DEAD-box helicase) with its enhancer eIF4B, eIF4E (responsible for the 5'-cap binding), as well as eIF4G, a scaffolding protein.⁴⁻⁹ Meanwhile, mRNA circularization takes place through a set of poly-A-binding proteins attaching to the poly-A tail and interacting with the eIF4G scaffolding protein.^{8,10} This interaction is hypothesized to mediate recycling of the translation machinery- once one

translation cycle is complete, the components come back to the m⁷G-cap and re-initiate translation.

Free ribosomes are recycled from previous translations' post-termination ribosomal complexes containing mRNA-bound 80S ribosome, deacylated tRNA, and eRF1.⁹ Initiation factors 3, 1, and 1A are responsible for dissociating these components; they remain attached to the 40S subunit preventing its re-association with the 60S. As well, these factors trigger the recruitment of the ternary complex (TC) of the initiator Met-tRNA_i^{Met}, an eIF2 GTPase and a GTP molecule, forming the 43S pre-initiation complex.^{7,9}

Finally, conformational changes in both mRNA and 43S -bound initiation factors facilitate the association of the 5' cap with the pre-initiation complex. This is a critical and a rate-limiting step leading up to the mRNA scanning.⁹⁻¹¹ eIF4F initiation complex promotes the 43S interaction with mRNA.⁷ Initiation factors 1, 1A and 4A, 4B and 4G facilitate the ATP-dependent unwinding of the secondary mRNA structures during scanning.

Upon start codon recognition, eIF5 binds eIF2 β subunit, activating eIF2 γ -mediated GTP hydrolysis.^{8,9,12-14} At this stage eIF2-GDP along with most of the other initiation factors is ejected. eIF2-GDP must be recycled into eIF2-GTP via eIF2B nucleotide exchange factor.⁷

GTP-dependent protein eIF5B GTPase promotes the recruitment of 60S ribosomal subunit and its joining with the 40S-Met-tRNA_i^{Met}, producing an 80S initiation complex.^{7,9}

Elongation process involves a codon-specific delivery of consecutive amino acid-charged tRNAs (tRNA^{aa}) by an elongation factor eEF1A to the ribosomal acceptor (A)-site. The reaction is catalyzed by GTP hydrolysis, upon which eEF1A-GDP complex is released to be recycled by the guanine exchange factors.¹⁵ Peptide bond forms between the tRNA-bound amino acid and

the preceding one. The tRNA is now deacetylated in a hybrid state between peptidyl (P) and exit (E) sites, while the following tRNA^{aa} is in a transient state between A and P sites.

Elongation factor 2 promotes translocation of the ribosome, positioning deacetylated tRNA in the E-site, the following tRNA in the P site and the mRNA itself with the next codon to be translated in the A site.¹⁶ Protein eEF3 promotes the release of deacetylated tRNAs from the E-site in an ATP-dependent manner as promoted by eEF3 and to be recharged with appropriate amino acids.¹⁷ Uncharged tRNAs are then aminoacylated by 3'-esterification with the corresponding amino acids catalyzed by aminoacyl-tRNA synthetase family of enzymes.¹⁸

Termination in the eukaryotic organisms is triggered by recognition of one of the three stop codons by release factor 1 (eRF1).¹⁶ The ester bond between resultant polypeptide and the P-site tRNA is hydrolyzed by peptidyl transferase, releasing newly-formed amino acid chain.

Essential protein eRF3 is suggested to trigger eRF1 dissociation from the ribosome complex.¹⁶

1.2 Alternative Methods of Translation Initiation

Various translation initiation control mechanisms exist in coordination with variations in cellular environment or specific formations in the mRNA structures. The most common methods of translation regulation are based on initiation factor availability. As a part of the stress response, translation of proteins required in bulk amounts in physiological conditions is down regulated via targeted degradation or inactivation of appropriate initiation factors. For instance, eIF2 subunit α may become phosphorylated by cellular kinases (such as SCN2, HR1, PERK).^{10,19} This interaction abolishes guanine nucleotide exchange factor activity, reducing the activity of the TC and suppressing the rate of cap-dependent translation.^{10,14,19}

Another translation-inhibiting strategy involves stress-induced dephosphorylation of eIF4E and 4E-BP1 (eIF4E binding protein 1).^{19,20} In unphosphorylated state, 4E-BP1 irreversibly binds

eIF4E region blocking its interaction with eIF4G. Upon phosphorylation, 4E-BP1 dissociates from its target leaving eIF4E free to bind the scaffolding protein.^{19,20} Further phosphorylation of Ser²⁰⁹ on eIF4E induces a conformational change enabling protein binding to the 5'-cap of the mRNA.²⁰ During cellular stress, the pathways leading to phosphorylation of the eIF4E - 4E-BP1 complex are repressed.^{10,19} In other cases, eIF4E and eIF4G undergo caspase-mediated cleavage, also inhibiting protein synthesis.²¹ However, evidence is present that as much as 10% of cellular mRNAs are commencing with translation during stress conditions.²²

Other mRNAs possess multiple upstream ORFs as a regulatory mechanism.⁷ For instance, *GCN4* gene activated during amino acid starvation possesses four upstream reading frames blocking its translation in non-hostile conditions.²³

1.2.1 Translation initiation via the ribosomal shunting

An alternative method has been studied in certain viruses allowing the initiation complex to bypass highly-structured mRNA regions that could cause stalling and ribosome dissociation.^{5,11} Ribosomes bind mRNA in a cap-dependent manner and scanning of the 5'-UTR commences until encountering a complex secondary structure, such as hairpin formation. This triggers ribosomal arrest and dissociation from the mRNA molecule. Initiation complex is then shunted downstream of the unpassable region and resumes scanning activity until encountering a start codon.^{5,24} The mechanism of this process is not well-understood. Peculiarly, the mRNA sequences acting as landing sites for the advanced initiation complex were found to be complementary to the 3'-proximal sequence of 18S ribosomal RNA.^{5,24}

1.2.2 Effect of leaky scanning on translation initiation and presence of uORFs

Certain eukaryotic mRNAs were found to possess several AUG start codons in their 5'-cap. Closer inspection showed these to vary in translation initiation efficiency based on their surrounding sequence context. In vertebrates, a Kozak sequence (5'-GCCGCC(A/G)CCAUGG-3') is recognized as a favorable translation initiation site.^{24,25} Initiation codons located in imperfect replicas of the Kozak sequences are less efficient as translation start sites. Thus, two variations of protein are being synthesized in different quantities²⁴.

Another method of translational control employs several ORFs upstream of the actual protein-coding sequence.^{7,24} For instance, Gcn4p, protein governing amino-acid synthesis under starvation, is controlled by 4 uORFs.⁷ Under optimal conditions 80S initiation complex is formed and translation of the first uORF takes place, but 40S ribosomes remain attached to the mRNA and re-initiate translation of the subsequent ORF downstream upon recruiting the essential ternary complex.⁷ In optimal physiological conditions TC is abundant and is readily recruited for translation of the following uORF sequence. Upon termination, translation machinery dissociates fully before reaching the protein-encoding ORF, leaving the gene product repressed. In conditions of cellular stress, such as nutrient or amino acid starvation, the TC component eIF2 is inactivated by phosphorylation, rendering TC inactive, and its recruitment inefficient.¹⁵ In this scenario, a functional TC complex is likely to be recruited only after the last regulatory ORF has been bypassed by the cap-scanning complex, allowing re-initiation at the coding ORF and translation of the gene product.^{7,24}

1.2.3 Internal ribosomal entry sites (IRES) - mediated translation initiation

The modes of translation discussed so far involve translation machinery binding mRNA cap. In an alternative mechanism, ribosomes and associated factors bind mRNA in the vicinity of the

start codon without scanning the 5'-UTR. Originally such interactions were discovered in viral systems, explaining how invading genome was expressed when infected cells' defense systems shut down general translation. As early as in 1990s, however, researchers started coming across eukaryotic mRNAs exhibiting similar characteristics. They found this mode of translation initiation was mediated through a *cis*-acting highly-structured 5'UTR.²⁶ These regions were termed Internal Ribosomal Entry Sites (IRESs).^{6,10,14,27} It is now hypothesized that 10-15% of cellular mRNAs are IRES-regulated.^{24,28}

Recent studies found that IRES-mediated translation is implicated in particular in genes controlling cellular stress response, life cycle and survival.^{10,14,19} These include *c-myc*, Apaf-1, p53, XIAP, VEGF, HSP90 and c-Src, all involved in some way in cell cycle control and implicated in cancer.^{10,19,29} It appears that canonical, cap-dependent translation is responsible for proteins required by the cell in bulk amounts, while IRES is responsible for certain essential proteins required in smaller quantities and whose expression is under tight translational control.²⁴

Multiple mutagenic tests were performed on IRES sites to identify regions crucial to initiation activity.^{13,14,30} Although this method was successful in abolishing activity of specific transcripts, no particular consensus sequence has been identified between all IRES-containing mRNAs. However, most known IRES-regulated genes share certain characteristics.^{5,10,14,26,30} They are mostly induced in various conditions implicated in cellular stress, such as amino acid and nutrient starvation, hypoxia, mitosis, heat shock and viral infection.²⁴ Such genes may be completely suppressed in normal cellular environment, but are expressed under the threat of apoptosis. It may also be vital for some genes to be expressed continuously; these can switch between cap-dependent translation initiation and IRES when canonical means of translation are

abrogated.²⁴ Another feature of many IRES-containing genes is long and highly structured 5'-UTRs.^{10,24} As well, they tend to have several AUGs apart from their genuine translation start codon.²⁴

Several types of IRES have been defined so far, requiring different elements for efficient activity.⁸ For instance, some genes' mRNAs can bind 40S-eIF2-eIF3-eIF5 complex, which is necessary and sufficient for that particular IRES to initiate protein expression.⁸ A number of elements have been identified, mainly mRNA-binding proteins, that enhance IRES activity. Named IRES-trans-acting factors (ITAFs), these are peculiar in having various other roles in the cell.¹⁰ Many ITAFs belong to the group of heterogeneous nuclear ribonucleoproteins shuttling between nucleus and the cytoplasm; they are also implicated in mRNA splicing and export. It has been hypothesized that ITAFs have a role in remodeling IRES structures, bridging them with ribosomes and other proteins essential to the process, in some ways even replacing the canonical initiation factors.¹⁰ For instance, during cell cycle regulation, ITAF hnRNP C1/C2 positively regulates translation of yet another cellular ITAF unr. The latter, in turn, stimulates IRES-mediated translation of protein PITSLREp58 actively involved in spindle formation.^{10,26} As well, a polypyrimidine-tract-binding protein (PTB) happens to be one of the most common ITAFs, while also having a role in translation, mRNA stability and splicing. It is required for most of the known cellular IRESs, in particular those involved in apoptosis.²⁶

Other genes require different combinations of canonical and non-canonical initiation factors to function efficiently.²¹ Yet another subset of the IRES-containing mRNAs seem to rely on the actual structure in their 5'-cap that is complementary in sequence to the 18S ribosomal RNA; IRES appears to recruit the initiation complex through the Shine-Dalgarno-like interaction.¹⁰

1.3 Importance of stress response to translation and the role of Hsp90p isoforms

As mentioned previously, IRES-mediated translation becomes preferable during the situations when canonical initiation is compromised. In sub-optimal conditions production of most proteins is shut down. Only expression of those proteins maintaining cell survival, passage through a particular life stage or controlled death of the cell is upregulated, often through alternative modes of translation initiation, including IRES. Activation of these is reliant on specific modifications to positive and negative regulators and ITAFs, such as phosphorylation, subcellular localization and alteration of their expression levels.¹⁰

Circadian rhythm oscillations, found in most organisms, are governed and temporally coordinated at transcriptional, translational and post-translational levels.³¹ Some of the genes involved are found to be under IRES control. *Period1* gene in mammalian hypothalamus is one of the well-studied regulators of the circadian cycle. Recently it was found to have an IRES site in its 5'-UTR; being positively regulated by a heterogeneous ribonucleoprotein hnRNP Q.^{31,32}

During mitosis, expression of proteins involved in the cell cycle progression is upregulated. In particular, cap-dependent translation is diminished during the G2/M transition.¹⁰ As a result, entire cellular cascades become dependent on alternative modes of translation initiation. One of the pathways involved is initiated by localization of an ITAF hnRNP C1/C2, which stimulates IRES-mediated translation of another ITAF urn. The latter enhances IRES-mediated translation of the protein PITSLREp58 involved in spindle formation.^{10,33}

IRES-mediated translation is found to be regulating various factors involved in oncogenesis and tumor suppression. As noted earlier, being active when cap-dependent translation is repressed makes it a convenient method of controlled expression of genes deciding cell fate. Tumor suppressor *TP53* is known to produce an isoform ΔN -p53 via IRES-mediated translation

specifically during stress conditions, such as endoplasmic reticulum stress and DNA damage in specific cell types.^{34,35} It has been speculated that mutations in the 5'-UTR contributing to IRES activity may weaken the interactions between the essential initiation factors and the mRNA, resulting in decreased p53 expression.³⁵

It was found that proteins controlling cell fate during various stress conditions are under IRES control. For instance, X chromosome-linked inhibitor of apoptosis (XIAP) and Bcl-x_L proteins are both pro-survival factors. XIAP is responsible for inhibition of caspases 3, 7, and 9, while Bcl-x_L stabilizes homeostasis within the mitochondrial membranes.^{10,22} Messenger RNAs of both proteins have been shown to contain IRESs negatively regulated by an ITAF tumor suppressor programmed cell death 4 (PDCD4).^{14,22} The latter, in turn, is under control of a ribosomal protein kinase 6 (S6K2) that is phosphorylated by the anti-apoptotic fibroblast growth factor 2 (FGF-2). In cancerous cells, activated S6K2 phosphorylates PDCD4, resulting its cleavage and derepression of XIAP and Bcl-x_L IRESs.²²

Heat shock proteins as a class are chaperones assisting with protein folding and association. Although ubiquitously expressed in physiological conditions, they become particularly important during cellular response to various stressors, when sub-optimal intracellular conditions hinder protein forming functional tertiary structures.^{36,37} As such, several studies implicated IRES-mediated translation as a regulatory mechanism for heat shock protein expression.^{10,38,39} Several classes of heat shock proteins exist, HSP90, in particular, has been implicated in apoptotic and necrotic responses to certain types of stress.³⁸ The chaperone acts in form of a dimer and is ATP-dependent.⁴⁰ In *S. cerevisiae* this class consists of two paralogs that arose due to whole genome duplication.⁴¹ HSC82 (YMR186W) is abundant in cells in optimal conditions, while HSP82 (YPL240C) is upregulated during stress.^{37,38} Despite having 89%

homology at the nucleotide level and 97% homology at the amino acid level, the two isoforms have antagonistic functions.^{37,38} Overexpression of Hsc82 confers resistance to acetic acid treatment, while overexpression of Hsp82 promotes apoptosis under the same conditions.³⁸

1.4 IRES mediated translation in human health

In humans, deregulation of IRES elements in essential genes due to mutations result in serious health conditions. For instance, a C>T transition in the 5'-UTR of a specific variant of connexin-32 results in loss of IRES-mediated translation of the gene product in the nervous system.⁴² The lack of protein is a contributing factor to an X-linked form of a Charcot-Marie-Tooth disease.⁴² In a less-studied illness, the fragile X syndrome, an expanded CGG element in the FMR1 gene transcript is a part of an IRES. The protein product is not translated in affected individuals, though the mechanism of regulation has not been identified.⁴²

One of the characteristics of cancer is deregulation of normal cellular pathways. This can be achieved by mutations in sequences regulating gene expression on transcriptional and translational levels. Recently, alterations were found in important cell fate-determining genes governed by IRES elements. For instance, alteration in expression of the c-myc transcription factor has been linked to many types of cancer. Of importance to this study is the C>T transition mutation in the IRES segment of the c-myc cap. It results in an enhanced binding of an ITAF hnRNPK, upregulating protein expression, which has been associated with multiple myeloma.⁴²

Another IRES-containing oncoprotein, c-Src, has a direct impact on translation in stressed cells.^{2,10,19} In normal environment it is involved in cell cycle progression and apoptosis. During stress, it is expressed through IRES-mediated translation independently of eIF2 α and ternary-complex.^{10,19} As mentioned previously, one of the methods of translation repression during stress involves blocking initiation factor 4E through dephosphorylation of itself and its

inhibitor. C-src is able to alleviate the constraint by re-activating necessary kinases and restoring translation initiation.¹⁹ Increased expression of c-src has been associated with uncontrolled cell differentiation, angiogenesis and metastasis, which are important characteristics of tumorigenesis.¹⁹

Normal expression heat shock proteins during carcinogenesis may actually aid in tumor survival by stabilizing the folding of inappropriately-expressed proteins.³⁶ As well, deregulated expression of HSPs and mutations in their clients may alter the pathways normally promoting apoptosis, which would normally result from imbalanced signaling.^{36,38} Current chemotherapeutic research is aimed at elucidating the exact mechanisms of HSP function to develop HSP inhibitors as a potential cancer treatment.^{36,43}

1.5 Systems Biology and Functional Genomics

Aside from a traditional approach of studying functional properties of one gene product at a time, one can look at the entire cellular system as a whole. This approach allows one to analyze the big picture - the cross-talk between various pathways and their implication on the cellular phenotype. The methodologies required to elucidate gene functions in a context of an entire cell are collectively referred to as functional genomics methods.⁴⁴ The focus of functional genomics is on cellular processes and interactions as opposed to structural or sequence information. Large-scale studies are employed to generate vast amounts of data that can then be analyzed to elucidate both individual proteins' functions, and to understand the interconnected networks and the functional association of genes in a living cell.^{45,46} Several approaches have been developed to collect this kind of data, such as large-scale yeast-2-hybrid screens, various microarrays and extensive tandem affinity purification (TAP)-tagging combined with mass

spectrometry. Large amounts of information collected by these methodologies can be analyzed through sophisticated bioinformatics software leading to further developments in the field.^{2,47}

Such large-scale studies have been used to outline many important interactions. For instance, due to newly-recognized abundance of the non-coding RNAs (rRNAs, snoRNAs, snRNAs, etc.) along with the traditional ones (rRNA and tRNA) in cellular systems and an unusually large number of conserved proteins processing them alongside the ribonucleoproteins (RNPs), a study in 2004 was performed in an attempt to elucidate the complex network of these processes and clarify the functional significance of the proteins involved.⁴⁸ A large-scale examination of RNP complexes and associated RNAs was conducted through TAP-tagging of known proteins involved in RNA biogenesis and processing and pulling out their associated factors.

Researchers followed-up with the large-scale systems to identify and quantitatively measure the abundance of novel RNPs and stably-associated RNAs. Two variations of mass spectrometry (MS) were used, modified to ensure less abundant proteins are pulled out and to quantify the stoichiometric proportions of all factors in a complex. Microarray was performed to identify non-coding RNAs associated with the pulled-out protein complexes. The study identified 56 complexes and 36 additional single proteins involved in RNA processing and RNP function, some of which were previously characterized associations, validating their methods, and others novel elements augmenting the RNA processing network. As a benefit of the quantitative methods employed, researchers were also able to identify stoichiometric proportions of the different proteins and complexes. Apart from establishing functional interactions of the identified proteins, a variety of RNA elements associated with protein complexes were found, suggesting that non-coding RNAs may be important for the RNP complex function. Overall, this investigation successfully employed several large-scale methodologies to establish

extensive network of RNA-processing pathways and elucidate functional significance of its elements.⁴⁸

It has long been known that a certain degree of functional redundancy exists in cellular pathways.^{49,50} For example, human genome encodes a much smaller number of proteins than originally predicted.^{51,52} Alternative splicing of mRNA transcripts is an important mechanism of increasing protein diversity within the cellular system. Another approach to expanding the productivity without increasing the number of functional units is to diversify the capabilities of proteins, enabling them to function in different pathways. Certain proteins in optimal cellular conditions perform known roles, while during mitosis or induced stress become recruited in pathways that may be analogous or completely unrelated from the original function.^{10,51,52} The process of translation is no exception. IRES-mediated initiation is ultimately governed by cellular requirements, often manifested in the availability of specific components required for translation initiation. Such functional redundancy in biological systems has a buffering effect, preserving phenotypic stability against spontaneous or induced mutations.^{53,54} At the same time they maintain genetic variation, allowing for species' survival of the perturbations in the environment.⁵³ Therefore, if one protein is inhibited from function, its final outcome may be rescued by a different pathway, resulting in the healthy phenotype of the cell.

Such functional overlap of the two proteins implies genetic interactions between them.⁵⁴

Whenever phenotypes of double mutations in the two genes cannot be explained by the additive phenotypes of the single mutations of these genes, they are said to be genetically interacting. For example, deletion of gene X in *S. cerevisiae* does not have any effect on the phenotype. Deletion of gene Y reduces cell growth by 5%. However, a double XY mutation reduces the growth by 70%. Such detrimental consequence cannot be explained by the additive

effects of single deletion mutants. This result implies that the two gene products may compensate for one another. In a strain with deletion of gene X, protein Y performs its original function and takes over for the missing product and vice versa. The concept has been extensively used to study gene function in yeast and adapted for studying functional genomics in other model organisms including *Caenorhabditis elegans* and *Drosophila melanogaster* species.^{49,55–58} Homologous genes and analogous interactions can eventually be investigated in humans.

In this study we chose *S. cerevisiae* as a model for simplicity of growth and ease of genetic manipulation (yeast being able to survive in both haploid and diploid states), as well as a high degree of conservation between yeast and humans. Since mis-governing of IRES-mediated translation, like other regulatory mechanisms, may be implicated in serious health conditions, it is important to be able expand this research onto mammalian systems in the near future.

1.6 Objective of the study: identification of novel candidates involved in IRES-mediated translation initiation

As mentioned previously, genes containing IRES in their 5'-UTR are implicated in cell cycle control, stress response, and induction of apoptosis. Misregulation of any of these processes may lead to important human conditions such as onset of cancer.^{9,19,59} Therefore, it is not surprising that a lot of research is aimed at elucidating the precise mechanisms of IRES-regulated translation as well as the details of factors that affect this process. In *S. cerevisiae* only about 20% of the genome is essential for survival of the organism.^{54,56} Deletion of the remaining genes does not produce a lethal phenotype suggesting a high degree of functional overlap for various gene. In addition about 25% of the yeast genes are orphans and have no well defined function or cellular process in which they participate.⁶⁰ In addition, the list of genes with novel

function during translation continues to grow.²⁶ Therefore, we hypothesize that there remain many elements involved in IRES-mediated translation initiation that are still unidentified. This study is directed at identifying novel genes involved in IRES-mediated translation and investigating their interactions within the translational process. For this, yeast gene deletion array is used to study the effect of a loss-of-function (gene deletion) on IRES recognition using a reporter constructs.

CHAPTER 2. MATERIALS AND METHODS

2.1 Vectors

Plasmids containing IRES from genes *HSP82* [YPL240C], *HSC82* [YMR186W], and *CLN3* [YAL040C, a -ve IRES] were a kind gift from Dr. Martin Holcik. All three plasmids contained ampicillin and canavanine resistance genes as well as *URA3* gene conferring cells the ability to grow on uracil-deficient media. As well, vectors possess a hairpin in their 5'UTR abolishing canonical translation initiation and a *lacZ* reporter gene fused downstream of the IRES element (Fig.2.1). Cultures with gene deletions having an effect on IRES activity will exhibit little or no β -galactosidase activity in presence of X-gal and ONPG. Candidate gene overexpression vectors were obtained from a Fisher Yeast MATa ORF Collection (YSC3868 in *E.coli* and YSC3870 in *S. cerevisiae*) (Fig.2.2). Plasmids were isolated from *E. coli* using Invitrogen Purelink Quick Plasmid Miniprep Kit (Ref# K210010). From MATa yeast vector isolations were performed using Promega Wizard® Plus SV Minipreps DNA Purification System (Cat.# A1460). Plasmids were transformed into *S. cerevisiae* MAT α using Lithium Acetate (LiAc) method. Concentrations of obtained plasmids were measured using ThermoFisher Nanodrop.

2.2 X-gal Lift Assay

A set of haploid yeast colonies containing a single deletion and *LacZ* reporter plasmid was grown at 30°C (Fisher Scientific Isotemp Incubator, Model 655D) for 2 days on YPD media(2% agar, 1% yeast extract, 2% peptone, 2% glucose) (uninduced control) as well as YPD media containing 2% galactose in place of glucose to induce the expression of the *LacZ* gene. The colonies were then lifted off the media onto a nylon membrane (GS Healthcare Amersham Hybond™ N⁺ Membrane CAT# RPN303B) and subjected to a liquid nitrogen treatment for 30 seconds. Membranes were then unthawed for 15 seconds and placed on top of 2 filters (Fisherbrand Filter Paper P3 Cat.# 09-802-1A) in a prepared solution of 4mL Z-buffer (16g

Figure 2.1. Construct model used for the X-gal and β -gal experiments to identify IRES-influencing candidate genes. Galactose-induced promoter is followed by canonical expression-inhibiting harpin structures. IRES structures are fused to the LacZ reporter. URE3, ampicillin and canavanine resistance markers are used for selection.

Figure 2.2. Model of an overexpression construct used in this study. Open reading frame (ORF) of interest is under control of a galactose-inducible promoter *GAL1*. *URE3* and ampicillin resistance markers are used for selection.

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 5.5g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.75g KCl, 0.246g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter, pH 7), 20 μL of 2% X-gal (2% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside in dimethyl formamide; BioShop, cat.#: XGA001.1) and 8 μL β -mercaptoethanol. Membranes were incubated at 30°C overnight and scored for change in colony color. If the gene has positive influence on IRES-mediated translation, the colony with corresponding deletion will be white, since β -galactosidase protein cannot be expressed and would not react with the X-gal substrate to produce blue colour.

2.3 β -galactosidase Assay

Candidate yeast strain with a β -galactosidase reporter construct was inoculated in 5mL complete medium lacking uracil + galactose (per liter - 6.7g yeast nitrogen base without amino acids/with ammonium sulfate, 2g amino acid mixture lacking uracil, 2% galactose) in triplicates and grown at 30°C on 200rpm shaker (New Brunswick Scientific NOVA40 Shaker Series) overnight. After incubation cells were centrifuged (Eppendorf Centrifuge 5804R [15amp]) at 4000rpm for 4min at RT and resuspended in 3mL of Z-buffer and resuspended by vortex briefly. Samples were then centrifuged as above and Z-buffer wash repeated. After 2nd round of centrifugation the cells were resuspended in 0.5mL of Z-buffer and kept at 0°C.

OD₆₀₀ of the samples was measured (1:10 ratio of sample : Z-buffer) with Z-buffer as a blank. 200 μL of cell samples were combined with 800 μL Z-buffer, 20 μL 0.1% SDS and 50 μL chloroform in a new tube and mixed for 15 seconds each. Resultant cultures were then heated at 30°C for 15min and combined with pre-warmed 200 μL ONPG (4mg/mL, BioShop, cat.#: ONP301.1), then mixed by vortex for 5 seconds and incubated at 30°C until development of colour from clear to yellow. Upon color change the reaction was stopped with 500 μL of 1M Na_2CO_3 and samples were centrifuged at 5000rpm for 4min at RT. OD₄₂₀ and OD₅₅₀ of the supernatant were then measured (solution of 2:1 z-buffer : 1M Na_2CO_3 used as a blank).

β -galactosidase activity was then measured as follows:

$$\text{Activity Units [A.U.]} = 1000 \times (\text{OD}_{420} - 1.75 \times \text{OD}_{550}) / (t \times V \times \text{OD}_{600})$$

2.4 3-aminotriazole and antibiotic sensitivity assays

Strains were incubated in YPD with 220mM acetic acid for 60 and 200 minutes before being spotted on solid YPD media alongside the controls (untreated cultures) in dilutions of 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} . All strains were also spotted on media containing 35mg/L 3-aminotriazole (3-AT). All trials were produced in triplicates and incubated at 30°C for 24 hours.

2.5 Query strain construction

Query strains consisted of genomic deletions of the candidate genes in the mating type α of *S. cerevisiae* obtained by the gene knockout method.

A set of primers was designed flanking the region approximately 100bp upstream of the gene of interest (forward primer) and 100bp downstream (reverse primer) (Fig.2.3). The primers were designed such that the 3' ends overlapped the gene conferring resistance to nourseothricin (*NatR*). A plasmid pAG25 was kindly provided by the members of Dr. Golshani's lab containing a full *NatR* gene. PCR was performed isolating the full gene fragment from the vector and resulting in *NatR* flanked by the 5' and 3' fragments of the gene of interest. The construct is then transformed into yeast mating type α by Lithium Acetate method. Cells were spread on the YPD media containing 100mg/L clonNat to select for those that integrated the *NatR* through homologous recombination, thus replacing the gene of interest. Confirmation primer was designed 1000 base pairs or less upstream of the gene of interest (Fig.2.3). MAT α transformants

Figure 2.3. Primer design for gene knockout and verification. Forward and reverse primers (P_{fwd} and P_{rev} , respectively) were designed to overlap nourseothricin resistance (Nat^R) gene and the gene to be replaced. Nat^R segment was isolated by PCR using P_{fwd} and P_{rev} from pAG25 vector. It was then transformed into MAT α yeast strain. Transfectants were confirmed to have incorporated the insert into their DNA by growth on clonNAT antibiotic as well as by verification PCR using confirmation primer up to 1000 nucleotides upstream of the gene (P_{conf}) and a sequence inside the Nat^R gene (P_{nat}).

were then verified by colony PCR using confirmation primer and a sequence located inside the *NatR* gene. All primer sequences used are listed in Appendix I.

2.6 PCR conditions

Reactions were constructed as outlined in table 2.1. Reaction volume of 50 μ L was used for creation of insert for knockout, 20 μ L reaction volume was used for colony PCR confirmation. For the colony PCR, a single colony was resuspended and grown in 1mL of liquid YPD with agitation at 30dC 4-12hrs. Every sample was then centrifuged to a pellet (13.3xg for 1min) and resuspended in 1mL of milliQ H₂O. The washing procedure was done twice, before final resuspension of the cells in 1mL of milliQ H₂O. 1.25 μ L of the resultant culture was used as a template in the colony PCR reaction. Thermocycler conditions used for all PCR reactions are outlined in table 2.2. PCR product purification was done using GE illustra GFX PCR DNA and Gel Band Purification Kit (cat#: 28-9034-70).

2.7 Lithium Acetate *S. cerevisiae* transformation

Vectors and gene replacement inserts were transformed into yeast strain by chemical transformation. MAT α was grown in 5mL of liquid media with agitation for one night at 30°C. 3mL of the resultant culture was transferred into 40mL of fresh media and grown at 30°C with agitation for 2-3 hours until $0.8 > OD_{600} > 1.2$. Culture was then centrifuged at 3000rpm for 2 minutes and resuspended in 10mL of milliQ water. In a same manner cells were washed with 1mL of 0.1M LiAc (Sigma cat#: L6883) and finally resuspended in 200 μ L of 0.1M LiAc.

Up to 1000ng of DNA of interest was mixed with 10 μ L Salmon Sperm single-stranded DNA (Invitrogen cat.# 15632011), 240 μ L of 50% PEG (Bioshop #PEG335.500), and 36 μ L of 1M LiAc. 50 μ L of cells was used per transformation. Cultures were incubated at 30°C for 60min and

Table 2.1. Concentrations of reagents used for PCR reactions. For colony PCR 20 μ L final reaction volume was used. 50 μ L reaction volume was used for creation of the insert for knockout.

Reagent	50 μ L rxn volume (μ L)	20 μ L rxn volume (μ L)
milliQ H ₂ O	38	15.2
10X PCR buffer [Invitrogen Cat.#1005581]	5	2
50mM MgCl ₂ [Invitrogen Cat.#985288]	1.5	0.6
10mM dNTP mix [Amresco Cat.#1932C175]	1	0.4
100mM Forward Primer	1.25	0.5
100mM Reverse Primer	1.25	0.5
Template DNA	1.25	0.5
Taq DNA Polymerase [BioShop Cat.# 3L31598]	0.75	0.3
Total	50	20

Table 2.2. Thermocycler conditions used for all PCR reactions.

Step	Temperature °C	Time	Cycle
1. Denaturation/Cell lysis	95	5 min	
2. Denaturation	95	45 sec	
3. Annealing	48	45 sec	
4. Extension	72	3 min	Step 2 - x35
5. Final Extension	72	2 min	
6. Hold	11	pause	

resuspended every 20min. Then 40 μ L of DMSO (Bioshop #DMS555.500) was added to each tube and after resuspending cultures were incubated on 42°C heat block (Baxter Multi-Blok Heater H2025-1A) for 15 minutes . Cells were then centrifuged at 8000rpm for 2 minutes, resuspended in 110 μ L liquid media and plated on agar. Cultures were incubated for 2-3 days at 30°C.

2.8 Agarose gel electrophoresis

Colony PCR reactions were run on a 1.1% agarose gel (Bioshop #AGA001) containing 0.2mg ethidium bromide in 1X TAE buffer (from 50X: 242g Tris Base, 57.1mL Glacial Acetic Acid, 0.5M EDTA, pH8). Electrophoresis was performed using VWR Accu Power 300 power supply system for 45 minutes at 80V. 5 μ L standard ladder was loaded per gel.

2.9 Synthetic genetic array (SGA)

Synthetic genetic array was performed as previously described by Tong and Boone.^{49,50}

Translation array in 384-colony format from Golshani lab was supplemented with other gene deletions researched to be relevant for translation. All deletions were in MATa strain with 230 random deletion colonies included as a background control. A border of histidine deficiency resistance gene *HIS3* deletions was placed on the periphery of the plates to ensure all tested deletions have access to the same amount of nutrients and difference in fitness is attributed to purely genetic differences.⁵⁰

A 384 floating pin E-clip style manual replicator (NUNC™ product #250393) with Microwell® Plate Copier (NUNC™ product #250539) was used for all replications. To replicate colonies on fresh media, the copier base is aligned over a source colony plate. The replicator leading pins are aligned with the holes in the copier base and the replicator is lowered with pins making contact with corresponding colonies. The replicator is then lifted off, the copier base is moved

over the fresh plate. Copier is aligned and lowered again with pins touching the agar and transferring the colonies onto the fresh media.

The finalized translation array was replicated and grown at 30°C on YPD+G418 (YG: YPD + G418 200mg/L) media for 2 nights just prior to mating. The query strain was grown in liquid 5mL YPD at 30dC with agitation overnight, then poured over solid YG and grown at 30°C for another night. Replicator was then used to pin 384-colony library of the query strain to be used for mating on YG media, which was grown at 30°C for 2 nights.

For mating, fresh DMA was pinned on top of the query strain on YPD and left in a plastic bag at room temperature for 2 nights. To select for MATa/ α zygotes, the colonies were then pinned on YPD with 200mg/L G418 and 100mg/L clonNat and grown for 2 days at 30°C. Sporulation was then induced by growing colonies at 22°C on enriched sporulation media (ESM: 10g CH₃CO₂K, 1g yeast extract, 0.5g glucose, 0.1g amino acid supplement powder for sporulation, 20g bacto agar, 50mg/L G418) for 8 days. Spores were selected to obtain MATa progeny on SD-His/Arg/Lys+cana/thia (synthetic dropout media: 1.7g yeast nitrogen base without amino acids or ammonium sulfate, 2g amino acid supplement powder -His/Arg/Lys, 20g agar, 50mg/L canavanine and 50mg/L thialysine); grown at 30°C for 2 nights. Second round of selection was performed on the same media for 2 nights at 30°C. Colonies were further subjected to selection on SD/MSG-His/Arg/Lys+cana/thia/G418 (synthetic dropout media: 1.7g yeast nitrogen base without amino acids or ammonium sulfate, 2g amino acid supplement powder -His/Arg/Lys, 20g agar, 50mg/L canavanine and 50mg/L thialysine, 200mg/L G418). At last the set was pinned on SD/MSG-His/Arg/Lys+cana/thia/G418 with 100mg/L clonNat and incubated at 30°C for 2 days.

2.10 Synthetic Dosage Lethality (SDL)

SDL is a modification of the synthetic genetic array, aimed at incorporating a vector over-expressing a gene into the deletion set. This methodology was also used to transform IRES vectors into the gene deletion array to identify candidates affecting IRES. MAT α strains were transformed with a vector of interest (IRES or overexpression vector). The strain was grown in 5mL liquid synthetic complete medium lacking uracil (URA⁻) overnight at 30°C with agitation. Liquid culture was then poured over the solid URA⁻ plate and grown at 30°C overnight. A 384 floating pin E-clip style manual replicator was then used to pin the query strain on URA⁻ media and grown at 30°C for 2 days.

Deletion master array was pinned on top of the query strain on YPD and mated in a plastic bag at room temperature for 2 nights. MAT α / α zygotes were selected on URA⁺+G418 (UG⁻) media at 30°C for 2 days. Sporulation was then induced in resultant strains on the ESM media at 22°C for 8 days. Spores were then selected on the synthetic dropout media SD-HAU+Can (6.7g YNB without amino acids and without ammonium sulfate, 1g MSG, 2g amino acid supplement powder mixture lacking histidine, arginine, and uracil, 20% glucose, 50mg/L Canavanine) at 30°C for 2 days. The final round of selection was performed on SD/MSG-HAU+G418+Can (1.7g YNB (w/o amino acids, w/o ammonium sulfate), 1g MSG, 2g amino acid supplement powder mixture lacking histidine, arginine, and uracil, 20% glucose, 50mg/L Canavanine, 200mg/L G418) for 2 nights at 30°C.

2.11 Conditional SGA and SDL and phenotypic suppression analysis (PSA)

The complete SGA and SDL arrays were subjected to antibiotic and temperature treatments. SGA and SDL were grown at 30°C on YPD and URA⁻ media, respectively, as a control. Cells were then subjected to high-temperature treatment (37°C), acetic acid and antibiotics. Detailed

list of treatments can be found in Table 2.3. Note that double deletion cells are generally more sensitive to environmental perturbations than cells containing a single deletion and an overexpression vector. Therefore, antibiotic concentrations used are lower for conditional SGA than for conditional SDL/PSA. As well, growth sensitivity test of double deletions on 100mM acetic acid was omitted.

A spot test was done for phenotypic suppression analysis on media containing 35mM acetic acid in dilutions of 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} as before in triplicates. Cell cultures containing double deletions were transformed with an overexpression vector of the candidate gene deleted in the strain as described above. Growth was measured as a proxy for suppression of sensitivity when compared to the matching double deletion strain. Strains containing single deletions with the overexpression vectors were compared for suppression of sensitivity to temperature and chemical stress to corresponding single deletion cell cultures.

2.12 Gene Ontology analysis

Gene ontology analysis was performed using online resources. Individual gene functions were researched through the *Saccharomyces* Genome Database (www.yeastgenome.org).⁶⁰ Genetic interactions and networks were analyzed through GeneMANIA (www.genemania.org).^{54,61}

2.13 Colony growth measurement and statistical analysis

Final colony size was measured via Growth Detector (GD) software as previously described.⁴⁴ Two-tailed t-test was performed.

Table 2.3. Treatments for conditional SGA, SDL and PSA. Cells were grown at 30°C unless otherwise stated. Measurements were taken after 24 and 48 hours of incubation.

SGA treatments	SDL treatments
YPD (control)	URA ⁻ (control)
YPD 37°C	URA ⁻ 37°C
YPD + 35mM Acetic Acid	URA ⁻ + 35mM Acetic Acid
YPD + 60mM Acetic Acid	URA ⁻ + 60mM Acetic Acid
YPD + rapamycin 2ng/L	URA ⁻ + 100mM Acetic Acid
YPD + paramomycin 10mg/L	URA ⁻ + rapamycin 4ng/L
YPD + cycoheximide 30ng/L	URA ⁻ + paramomycin 18mg/L
	URA ⁻ + cycoheximide 45ng/L

CHAPTER 3. RESULTS

3.1 Transformation of IRES-containing vectors into the essential deletion array of *S.*

cerevisiae

In order to identify the gene candidates with potential roles as ITAFs, large-scale screens were carried out where non-essential genomic deletions were examined for their ability to influence IRES-mediated translation initiation. Three vectors were used harboring a β -galactosidase reporter gene under control of the IRES-containing 5'-UTR from genes *HSP82* and *HSC82*, along with a control 5'UTR from the *CLN3* not containing an IRES. These were then transformed into yeast mating type α and pinned in a 384-colony array format (for an example see Fig. 3.1). Modified SGA protocol was employed to mate the ~5000 strain yeast non-essential deletion array with the resultant query cultures. Strains were then pinned on appropriate selection media, resulting in 3 deletion arrays, each harboring a reporter construct with *HSP82*, *HSC82*, or *CLN3* 5'-UTR. This resulted in the generation of ~15,000 transformed strains ready for large scale screening experiments.

3.2 Large-scale β -galactosidase reporter screening to identify mutants affecting IRES mediated translation

In order to determine the effect of each strain's deletion on IRES-mediated translation of the reporter protein, a large-scale screen encompassing all 15000 transformations was conducted. Colonies were lifted from the plate onto a membrane, burst by liquid nitrogen treatment, then exposed to the X-gal substrate that produces blue-colored product once β -galactosidase enzyme cleaves it. In this scenario, if the deletion in a particular strain has no effect on IRES function, reporter will be translated successfully and act upon the substrate, resulting in a blue colony. We anticipate that this would be the case for most of the colonies. If a deletion is essential for IRES function, the enzyme will not be translated, X-gal will not be cleaved, and the colony will

Figure 3.1. An example of the colony collection used in this study. Shown here are identical MAT α strains transformed with a vector over-expressing gene *VPS70*. According to research needs, every colony can be a different strain.

remain white in color (Fig.3.2). We observed that approximately 48 strains were white when transformed with *HSC82* - derived IRES vector. In contrast, only about 26 *HSP82* IRES - containing strains were white. Both *HSP82* and *HSC82* based screens were then compared to *CLN3* based screen to remove false positives. *CLN3* 5'-UTR contains no IRES, consequently if a colony remained white in both *CLN3* and one of the IRES screens, the result is likely due to extraneous effects in the system and not because of IRES activity. Comparing the data for *HSP82* and *HSC82* based screens the overlap between the transformants consisted of only two strain deletions: *DOM34* (*YNL001W*) and *ITT1* (*YML068W*). The list of the selected strains is found in Table 3.1.

3.3 Quantitative β -galactosidase reporter assay to identify mutants affecting IRES mediated translation

The exact degree of influence of each gene deletion on IRES activity was determined using small scale quantifiable β -galactosidase assay. Approximately 74 candidates were identified in the lift assay between the *HSP82* and *HSC82* - derived IRES screens. These were subjected to the liquid β -galactosidase assay, using ONPG compound as a substrate. The precise activity of the enzyme could be determined in this variation of the assay by measuring the amount of colour change resultant from the cleaved substrate.

Through the quantitative assay, 40 candidates were determined to have significantly depressed β -galactosidase activity of 0.5A.U. or less ($p < 0.05$) (Table 3.1). This indicates an efficiency of approximately 54% for our large scale screen to identify correct hits. As well, of interest are select strains, such as *PCS60*, *ERV29*, and *GAS2*, that actually demonstrated elevated cleavage of ONPG measuring greater than 1A.U.(Table 3.1).

Figure 3.2. X-gal lift assay membrane for deletion plate #5 containing HSC82-IRES-LacZ vector, demonstrating the differences in color changes (circled detail). The membrane was placed over live colonies grown on agar, then subjected to liquid nitrogen to induce lysis and placed in media containing X-gal substrate. Blue colonies indicate activated β -galactosidase reporter enzyme resultant from successful IRES-mediated translation, white colonies correspond to an intact substrate resultant from a failed translation of a reporter due to a deletion of an element essential for IRES-mediated translation. Colonies of questionable color change (*e.g.* bottom right hand corner) were not considered.

Table 3.1. ONPG-based β -galactosidase activity test results. HSP82 and HSC82 - derived IRES element controls the expression of *LacZ* gene. Decreased activity denotes the lack of an active enzyme in the strain due to deletion of a particular gene. β -gal activity is measured in activity units (A.U.) normalized to the wildtype strain. P-values were obtained using the two-tailed equal variance t-test.

<i>HSC82</i>			
Gene name	Normalized β -gal activity (A.U.)	Std. Deviation	P-value
WT	1	0.253664151	
YCR007C	0.001300428	0.000448896	0.0003077
MBA1	1.147571646	0.205842581	0.4151473
PCS60	1.637558353	0.30614999	0.0123674
FUN19	0.43692027	0.176183062	0.0114306
AFR1	0.000232047	0.000361182	0.0003057
ATP22	0.000562933	0.000145046	0.0003063
ATP5	0.004264752	0.001189315	0.0003134
RPP2B	2.07118E-05	2.05051E-06	0.0003053
SVF1	2.4896E-05	2.94243E-06	0.0003053
FCY21	0.84650753	0.178706382	0.3858049
CHC1	1.078655902	0.23302188	0.7827759
SEC28	0.006683697	0.011565832	0.0065445
LHS1	0.004913714	0.008492152	0.0064847
PTK1	0.498362148	0.115505845	0.098819
PLB1	0.000315282	7.34656E-05	0.0063352
YLR149C	0.523259442	0.120012977	0.1138372
MRPS16	1.36124E-05	2.17705E-05	0.0063257
TGS1	8.68451E-06	3.83771E-06	0.0063255
YPL067C	3.40096E-05	5.87202E-05	0.0005837
YDL012C	3.12654E-06	9.47801E-07	0.0005836
ZTA1	7.58782E-06	1.75426E-06	0.0005836
STP4	1.064728914	0.224547835	0.7420109
BUD3	0.425394587	0.047198154	0.0116541
GBP2	0.994664004	0.153786988	0.9769878
STF1	0.000211633	0.000240812	0.0005843
SEA4	3.83849E-05	7.7669E-06	0.0005837
BRR1	1.395338028	0.26530327	0.0459956
SGE1	0.827107188	0.018543752	0.2219431
YPR197C	0.799411943	0.172221927	0.2070948
YDR535C	0.004130835	0.003049099	0.0001133
RSM23	1.30518E-05	5.80546E-06	0.0001104
MSL1	1.017E-05	6.90822E-06	0.0001104
RPI1	1.20526E-05	4.48846E-06	0.0001104
PTC6	7.16389E-06	8.45388E-06	0.0001104
DOM34	0.000221862	0.000124307	0.0003043
THI2	0.656800516	0.258196855	0.0985431
ERV29	2.040112528	0.419541108	0.0021008
YBR232C	1.432096354	0.341328516	0.0664572
YOL106W	3.84065E-05	2.86814E-05	0.000304
IZH4	0.00071951	0.000240088	0.0003053
ADH6	0.001004048	0.000170573	0.0003058
YPR014C	7.9578E-06	9.33594E-06	0.0003039
RCR2	1.6058261	0.137887382	0.0037962
IRC8	0.425347075	0.071599039	0.0038258
LTP1	1.80845E-05	1.18493E-05	0.0001333
ITT1	2.34345E-05	2.07095E-05	0.0001333
CMC4	5.47783E-06	7.75123E-06	0.0001333
UGA4	0.0007947	0.000159183	0.0001339

<i>HSP82</i>			
Gene name	Normalized β -gal activity (A.U.)	Std. Deviation	P-value
WT	1	0.185557224	
YDR282C	2.31416E-06	1.25957E-06	4.2113E-05
RPL36A	2.75241E-06	1.25957E-06	4.2113E-05
CLB4	4.7253E-06	1.32304E-06	4.2113E-05
NUP2	5.94654E-06	1.48958E-06	4.2114E-05
TSA1	1.23684E-05	3.64743E-06	4.3721E-05
ALG8	0.00012016	3.01243E-05	4.3751E-05
URN1	8.3981E-06	7.91676E-06	4.372E-05
YBR064W	9.26105E-06	1.78738E-06	4.372E-05
NHP10	1.42254E-05	4.54443E-06	2.7169E-07
PET54	0.000819655	4.83078E-05	2.732E-07
RMD1	1.62174E-05	8.90077E-06	2.7169E-07
YBR062C	0.500456612	0.056473279	4.8561E-05
RPL37B	1.74116E-05	4.3696E-06	2.7169E-07
VTH1	0.011202466	0.001342915	2.9365E-07
AIM33	0.000190428	0.000283906	2.7202E-07
KTR3	1.32517E-05	2.67146E-06	2.7168E-07
DOM34	1.18546E-05	3.67453E-06	2.7168E-07
CAT2	0.000935679	0.000180864	2.7342E-07
YGR053C	6.34328E-06	1.59139E-06	9.8002E-05
YGR025W	3.29566E-06	2.26698E-06	9.8E-05
MF(ALPHA)2	0.910900031	0.151694639	0.54142094
SKM1	2.60525E-06	3.84918E-06	9.7999E-05
GAS2	0.846863043	0.220576845	0.34559217
PUF2	0.545887302	0.269973463	0.02671283
RPS1A	0.004462715	0.001575141	0.00010084
VPS70	0.001834915	0.000619598	9.9152E-05
ITT1	1.52424E-05	2.06735E-06	9.8007E-05

3.4 Measurement of the candidate deletion strains' sensitivity to 3-AT and acetic acid.

Further experiments were performed to analyze the candidate deletions' effect on stress-induced survival rate. IRES-mediated initiation is often activated during response to stress, contributing to translation of proteins essential for recovery. Therefore, if a gene required for IRES function is knocked-out, the resulting gene deletion strain may not synthesize an essential protein required for survival and hence may not be viable in the hostile environment. In this way the deletion strain for a gene that plays a role in IRES mediated translation may show sensitivity to certain stress conditions. Based on this information, we performed a spot test analysis on the target 40 gene deletion strains, examining their ability to grow in presence of 3-aminotriazole (3-AT) and acetic acid. 3-AT is an imitator of starvation by altering the pool of amino acids in the cell.⁵⁷ Acetic acid is shown to induce apoptosis by an unknown mechanism.⁶² Deletion of *HSC82* and *HSP82* are known to alter sensitivity to acetic acid treatment. Candidate strains were subjected to 220mM acetic acid for 60 and 200 minutes then spotted on YPD. For 3-AT sensitivity test, they were spotted on YPD containing 35mg/L 3-AT. As expected, cultures that showed sensitivity (measured by reduced or absent growth) to acetic acid treatment for 60 minutes were all sensitive to 200 minutes treatment (Table 3.2). Majority of strains that were sensitive to 3-AT also exhibited deficient growth in acetic acid (Table 3.2). The only exception is *DOM34Δ*, which was only affected by 3-AT.

3.5 Gene ontology analysis of candidate deletions and selection of strains for further study.

Gene ontology analysis was performed for the candidate gene deletions. As expected, the majority of the genes were already known to be implicated in various kinds of stresses providing further evidence for the competence of our screen. DNA damage response and replication stress was the biggest group (*ATP22*, *DOM34*, *RPL37B*, *RPS1A*, *STF1*, *STP4*, *TSA1*, *YDL012C*, *YLR149C*) (Fig.3.4). Next biggest group of genes was known to have roles in various

Figure 3.4 Functional overlap observed in gene ontology analysis for IRES-affecting candidates. Functional groups are colour coded as per legend. Majority of the genes have previously been implicated in stress response, consistent with the idea that IRES-mediated translation is induced during stress. There is a noticeable overlap between stress response genes and those involved in translation, vacuolar function and, unusually, mitochondrial translation. This is consistent with prior research, stating that a great degree of functional overlap is persistent through known ITAF elements.¹⁰

Table 3.2. Summary of properties of genes potentially involved in IRES-mediated translation. Color blocked cells indicate sensitivity to 3-AT or acetic acid. Gene deletions affecting IRES would fail to rescue their corresponding strains from stressful conditions induced by 3-AT and acetic acid. β -galactosidase induction is shown in normalized activity units (A.U.) for each isoform. Deletions of genes essential for *HSP82* or *HSC82* - specific IRES function would fail to translate the reporter, resulting in reduced amount of cleaved substrate and decreased β -galactosidase activity as compared to the WT. Note that only *DOM34* and *ITT1* exhibit diminished enzymatic activity for both. Summaries of associated functions highlight the most relevant processes implicating each gene. Prior research linked IRES-mediated translation to stress, genes with these functions were favored over others. Bold and underlined are candidates subsequently selected for follow-up screens based on all afore-mentioned data.

Gene Name [Δ]	3-AT	Acetic Acid		β -GAL TESTS [NORMALIZED A.U.]		Functions Associated
		60 min	200 min	HSP82	HSC82	
WT				1	1	
<u>ATP22</u>					0.00056	Mitochondrial translation, DNA replication stress
<i>BRR1</i>					0.13953	Splicing, 3'-end processing, snRNP biogenesis
<u>DOM34</u>				0.00001	0.00022	Ribosomal subunit dissociation, stress response, RNA cleavage in no-go decay, DNA replication stress
<i>GAS2</i>				0.84680		Spore wall assembly, UV damage, interaction with eEF2 and HSP40
<i>ITT1</i>				0.00002	0.00002	Translation termination
<u>MRPS16</u>					0.00001	Mitochondrial ribosomal protein, heat shock response, nutrient stress
<i>MSL1</i>					0.00001	Splicing,
<i>NUP2</i>				0.00001		Nucleoporin, chromatin organization, anoxia response
<i>PCS60</i>					1.63760	mRNA binding, oxalate degradation
<i>PET54</i>				0.00082		Mitochondrial translation
<i>PUF2</i>				0.54590		mRNA binding, transmembrane transport, vesicular trafficking
<u>RPL36A</u>				0.00000		Oxidative stress response, nutrient starvation, heat shock, translation
<i>RPL37B</i>				0.00002		Translation, DNA replication stress,
<i>RPP2B</i>					0.00002	Translation repression, translation elongation
<i>RPS1A</i>				0.00446		Translation, nutrient availability, DNA replication stress
<i>RSM23</i>					0.00001	Apoptosis
<i>STF1</i>					0.00021	DNA damage response, respiration
<i>STP4</i>					1.06470	DNA damage response, heat shock response
<i>TGS1</i>					0.00001	Nucleolar assembly, splicing
<i>THI2</i>					0.65680	Nutrient starvation
<i>TSA1</i>				0.00001		Oxidative stress, DNA replication stress, telomeric length maintenance
<i>UGA4</i>					0.00079	Vacuolar, nutrient starvation, heat shock response
<u>VPS70</u>				0.00184		Vacuole, splicing, calcium deficiency
<i>YBR062C</i>				0.50050		Unknown, filamentous growth pathway
<u>YBR064W</u>				0.00001		Dubious ORF
<i>YDL012C</i>					0.000003	Stress response, NHEJ
<i>YDR282C</i>				0.000002		Unknown, mitochondrial translation
<i>YGR053C</i>				0.00001		Unknown, stress resistance
<i>YLR149C</i>					0.52330	Unknown, cell cycle progression, DNA replication
<i>YPL067C</i>					0.00003	Unknown, stress response
<i>YPR014C</i>					0.00001	Dubious ORF

steps of translation (*RPL36A*, *RPL37B*, *RPS1A*, *GAS2*, *RPP2B*, *DOM34*, *ITT1*, *RPP2B*). These also included ribosomal genes.

Interestingly, four of the candidate genes were involved in mitochondrial translation (*ATP22*, *MRPS16*, *PET54*, *YDR282C*) (Fig. 3.4). Other important groups represented were splicing and vacuolar genes. The remainder of candidates were involved in various pathways some more important for regulation (e.g. cell cycle progression, *YLR149C*; apoptosis, *RSM23*) than others (cell wall assembly, *GAS2*). Many of these genes also had unknown functions.

Six genes were selected for further studies based on presence of sensitivity to chemicals, ontology and previous direct or indirect links to stress and translation (Table 3.2). These are *ATP22* (*YDR350C*), *DOM34Δ* (*YNL001WΔ*), *MRPS16Δ* (*YPL013CΔ*), *RPL36AΔ* (*YMR194WΔ*), *VPS70Δ* (*YJR126CΔ*), and *YBR064WΔ*.

3.6 Genetic interaction analysis

3.6.1 Synthetic sickness and lethality screen

In order to determine the validity of selected candidates as ITAFs and determine their potential roles in IRES-mediated translation, we decided to analyze their genetic interactions.

Genetic interaction refers to multiple genes interacting in overlapping pathways.⁵⁴ It does not presume the gene products are interacting physically, but rather it implies a functional relationship between the two.⁵⁴ Thus, genetically interacting partners can be linked to the particular processes in the cell, which helps with identification of these proteins' novel functions.⁴⁵ Since genetic interaction network of a protein exhibits direct links to the pathways it is involved in, large-scale genetic interaction studies provide valuable information for elucidation of the functional cross-talk in the system.

In many cases, under optimal environmental conditions, the lack of two nonessential genes produces no measurable phenotype. Specific conditions can be induced, depending on the study to encourage phenotypic manifestation of the genotype, and thus elucidate the roles of the genes in the system.^{49,54} These can take form of a chemical treatment (a drug affecting a pathway of interest, nutrient deprivation), or a physical change (temperature). Importantly, sub-inhibitory concentrations of drugs and temperature alterations should be applied to merely shock the system under study but not immediately induce cell death.^{49,54}

A large-scale method to systematically test genetic interactions is termed synthetic genetic array (SGA) method.⁵⁰ An array of strains with inactivated genes is used to generate a set of double deletions and evaluate their viability. The rate of cell growth is commonly quantitatively measured with cell lethality being the extreme phenotype. In this context the phenotype of double gene deletions that reduce cell viability and those that are lethal are termed synthetic sick and synthetic lethal, respectively. Colony size or optical density of cells (in liquid) with deletions in two genes is compared to that of a single deletion control strain.⁵³ On the basis of the function of the genes with which a target forms genetic interaction we can gain novel information about the function of the target gene. In this way, genes operating in the same cellular process will genetically interact with each other.^{48,57}

To further study the roles of the candidate genes in alternative translation initiation mechanism, we investigated the genetic interactions that they made with other translation genes. For this, an array of strains was collected with genomic deletions affecting translation in yeast mating type a (approximately 400 colonies). Next, in order to create a library of double deletions, we need to construct a set of strains in mating type α , containing genomic deletions of the six candidates. In this way the target gene deletion in mating type α can be mated to the array of single gene

deletion mutants of mating type a, to produce progenies with double gene deletions. Gene knockouts were done for each of the six selected candidates in yeast MAT α using lithium acetate method. Each transformation yielded between two and 20 colonies per plate. The identities of these mutant strains were confirmed by colony PCR. The lanes corresponding to the positive colonies had bands corresponding in length to the size of NatR gene (used to replace the gene of interest) and the region upstream of the replaced ORF (Fig.3.5).

Modified SGA method was used for the translation array and the candidate query strains to be mated and subjected to several rounds of selection as previously outlined. The result was six libraries, each containing a set of double deletions - translation deletion array that also carry a particular candidate gene deletion. Libraries harboring double deletions (one associated with translation array and the other for each of the six candidates) were grown in optimal conditions as well as induced by heat shock, or presence of sub inhibitory concentrations of acetic acid, cycloheximide, paramomycin, and rapamycin. By scoring reduction in colony size, we determined genetic interactions of each candidate under different growth conditions. As defined previously, if a double deletion exhibits sickness in optimal conditions or when subjected to some kind of stress, the genes in question are said to genetically interact. It should be noted that it is possible for the two genes to only interact under a specific condition such as

Figure 3.5. An example of an agarose gel electrophoresis analysis for 7 *VPS70* knock-out transformant candidates in a MAT α strain. Primers used for the PCR reaction are designed from a region upstream of the gene (fwd) and within the Nat^R cassette (rev), such that the band size corresponds to the total size of the amplified region; ~700bp for *VPS70*. Successful strains were then mated with the translation array to create libraries of double deletions used to identify the candidates' genetic interaction networks.

stress, as their functional relationship is only realized under such circumstances. In this way a genetic interaction is not observed under standard lab conditions but is only realized in a specific environment. In general, all six candidate genes were found to have interactions with genes that were structural components of the ribosomes (rRNAs and proteins) as well as various factors regulating ribosomal biogenesis (Tables 3.3, 3.4). Analysis of candidate-specific genetic interaction networks is as follows.

3.6.2 Gene ontology analysis of ATP22Δ genetic interaction network

When analyzing genetic interaction patterns, those of strain *ATP22Δ* in most experiments were found to be associated with structural ribosomal RNAs and proteins (*e.g.* Rapamycin, $p=1.13E-19$, Table 3.4). As well, it was connected to regulation of biosynthesis and processing of ribosomal components and their export from the nucleus (Tables 3.3, 3.4). The greatest colony size reduction in SGA was observed for two protein components of 40S ribosomal subunit *RPS6A* (74.59% size reduction, acetic acid) and *RPS27B* (70.56% reduction; acetic acid, cycloheximide, heat shock & paromomycin), as well as *SGS1* (YMR190C; 74.51% reduction; paromomycin) (Table 3.5). The latter is a DNA helicase involved in genome integrity maintenance, but has also been linked to the rapamycin response pathway.⁶³ Two nuclear genes were found to be associated with *ATP22Δ* in the general SGA screen - *NUP2* (52.80% size reduction in SGA) and *NUP84* (35.84% size reduction, SGA & acetic acid), that code for proteins involved in nucleopore complex and are implicated in the export of mRNAs from nucleus in response to heat stress ($p=2.17E-02$)(Table 3.3).

3.6.3 Gene ontology analysis of DOM34Δ genetic interaction network

DOM34Δ, apart from being associated with regulation of translation, has also been linked to regulation of structural components of the ribosomes and their precursors (Tables 3.3, 3.4).

Table 3.3. SGA analysis of the six candidates affecting IRES-mediated translation initiation. Data for SGA, acetic acid and heat shock conditional screens shown. GO annotations are included for the enriched functions with the associated p-values where available. Note the enrichment of ribosomal biogenesis and structural constituents throughout the treatments. Genetic interactions with these may link the candidates with translation mechanisms, relevant to IRES. Some unusual interactions are present, such as Swr1 complex with *VPS70Δ* and mRNA export from nucleus in response to heat stress for *ATP22Δ*.

	SGA		Acetic acid		Heat shock	
	GO	P-value	GO	P-value	GO	P-value
YBR064W	ribosomal small subunit biogenesis	1.76E-08	structural constituent of ribosome	1.07E-16	-	-
	ribosomal small subunit assembly	1.66E-03	small ribosomal subunit	9.96E-06	-	-
	rRNA processing	8.40E-03	ribosomal small subunit biogenesis	1.00E-03	-	-
VPS70	ribonucleoprotein complex assembly	1.94E-02	structural constituent of ribosome	1.80E-17	-	-
	ribonucleoprotein complex subunit organization	2.18E-02	small ribosomal subunit	2.45E-06	-	-
	Swr1 complex	1.38E-03	maturation of SSU-rRNA	4.82E-03	-	-
RPL36A	serine hydrolase activity	1.01E-02	mRNA catabolic process	1.31E-02	cytosolic large ribosomal subunit	2.60E-16
	structural constituent of ribosome	1.24E-02	deadenylation-dependent decapping of nuclear-transcribed mRNA	1.31E-02	cytosolic ribosome	2.60E-16
	cytosolic large ribosomal subunit	1.39E-02	ribonucleoprotein granule	2.47E-02	structural constituent of ribosome	9.53E-15
MRPS16	nucleosome organization	1.05E-02	ribosomal small subunit export from nucleus	4.28E-02	-	-
	polysome	1.49E-02	rRNA-containing ribonucleoprotein complex export from nucleus	4.28E-02	-	-
	-	-	ribosome localization	4.28E-02	-	-
DOM34	Translation regulation	2.25E-05	ribosomal small subunit biogenesis	8.84E-06	structural constituent of ribosome	1.87E-23
	-	-	rRNA processing	2.40E-03	ribosomal small subunit biogenesis	3.14E-03
	-	-	preribosome, small subunit precursor	4.29E-02	preribosome	7.39E-02
ATP22	RNA localization	6.23E-04	small ribosomal subunit	8.96E-25	structural constituent of ribosome	3.52E-31
	rRNA transport	3.15E-03	maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	1.51E-09	small ribosomal subunit	2.56E-18
	mRNA export from nucleus in response to heat stress	2.17E-02	small-subunit processome	4.61E-03	maturation of SSU-rRNA	2.52E-04

Table 3.4. SGA analysis of the six candidates affecting IRES-mediated translation initiation. Data for cycloheximide, paramomycin, and rapamycin conditional screens shown. GO annotations are included for the enriched functions with the associated p-values where available. Note the enrichment of ribosomal biogenesis and structural constituents throughout the table. Genetic interactions with these may link the candidates with translation mechanisms, relevant to IRES. Some unusual interactions are present such as enrichment of serine metabolic pathway in *RPL36AΔ* screen and Tor signaling in *DOM34Δ*. The link between IRES and stress response indicates these associations as interesting for follow-up.

	Cyclo		Paramo		Rapa	
	GO	P-value	GO	P-value	GO	P-value
YBR064W	structural constituent of ribosome	1.65E-28	structural constituent of ribosome	1.90E-23	structural constituent of ribosome	1.21E-06
	ribosomal small subunit biogenesis	3.43E-10	small ribosomal subunit	7.73E-13	RNA binding	3.19E-03
	maturation of SSU-rRNA	1.43E-06	maturation of SSU-rRNA	4.82E-05	-	-
VPS70	ribonucleoprotein granule	4.25E-04	structural constituent of ribosome	1.53E-04	ribosomal subunit	4.84E-12
	-	-	cytosolic small ribosomal subunit	1.37E-03	cytosolic small ribosomal subunit	5.86E-12
	-	-	small ribosomal subunit	1.31E-02	regulation of translation	1.55E-02
RPL36A	ribonucleoprotein granule	3.13E-04	ribosomal small subunit biogenesis	6.09E-07	structural constituent of ribosome	1.47E-03
	translational elongation	1.10E-02	maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	1.96E-05	cytosolic large ribosomal subunit	4.89E-03
	posttranscriptional regulation of gene expression	1.52E-02	regulation of translation	1.00E-03	serine family amino acid metabolic process	9.14E-03
MRPS16	cytosolic large ribosomal subunit	2.32E-06	structural constituent of ribosome	4.45E-31	structural constituent of ribosome	3.48E-27
	large ribosomal subunit	7.14E-06	maturation of SSU-rRNA	4.74E-07	cytosolic small ribosomal subunit	2.02E-11
	-	-	rRNA metabolic process	1.41E-06	small ribosomal subunit	2.72E-09
DOM34	structural constituent of ribosome	1.87E-23	structural constituent of ribosome	1.02E-48	structural constituent of ribosome	5.89E-02
	ribosomal small subunit biogenesis	3.14E-03	small ribosomal subunit	2.05E-22	regulation of translation	3.06E-01
	preribosome	7.39E-02	rRNA processing	2.05E-09	TOR signaling cascade	3.33E-01
ATP22	structural constituent of ribosome	1.40E-33	structural constituent of ribosome	1.01E-27	structural constituent of ribosome	1.13E-19
	maturation of SSU-rRNA	1.67E-06	ribosomal small subunit biogenesis	1.85E-07	small ribosomal subunit	9.96E-06
	rRNA processing	1.20E-05	maturation of SSU-rRNA	1.16E-05	ribosomal small subunit biogenesis	1.17E-02

Table 3.5. SGA screen data of *ATP22Δ*. Strain collection was created combining *ATP22Δ* with deletions of translation-related genes. Colony size was scored after growing resultant strains on plain YPD at 30°C (S), on YPD with 37°C heat shock (H), in presence of acetic acid (A), cycloheximide (C), paromomycin(P) and rapamycin (R). Percent size reduction is indicated for each gene. (*) symbols indicate DryGin data collected via similar experiments by Dr. Boone from University of Toronto.¹²¹ The greater is the reduction in colony size, the stronger genetic interactions between the two deleted genes are. Of particular interest were interacting partners showing %size decrease of above 70%.

<i>ATP22Δ</i>											
AAH1 ^S	29.58	GSY2 ^R	37.63	RPL13A ^C	34.45	RPS26B ^{A, C}	32.45	TGS1 ^{A, C, H}	39.77	YER086W ^{A, R}	43.49
AAP1 ^A	60.72	HAP4 ^R	35.65	RPL16A ^H	23.85	RPS27B ^{A, C, H, P}	70.56	TOR1 ^R	45.03	YGL232W ^R	42.86
ALB1 ^S	24.34	HPM1 ^{A, R}	32.21	RPL16B ^P	42.71	RPS30B ^C	32.63	TOS6 ^H	34.30	YJL056C ^{H, S}	30.28
BRR1 ^S	35.08	HTA1 ^C	34.88	RPL20B ^A	43.95	RPS4A ^A	27.09	TPK1 ^R	25.85	YJL160C ^R	55.68
BUD21 ^A	32.54	LTV1 ^S	33.23	RPL27A ^{R, A}	34.83	RPS4B ^{A, H}	64.76	TPK2 ^A	40.28	YKL074C ^A	52.69
BUD22 ^A	30.11	MCK1 ^A	29.63	RPL34B ^{A, R}	57.44	RPS6A ^A	74.59	TRP1 ^A	27.59	YKL205W ^C	46.94
BUD27 ^A	30.47	MHO1 ^A	23.39	RPL35B ^C	52.34	RPS7B ^A	38.55	TSA1 ^A	54.50	YLR085C ^S	57.99
CDH1 ^P	33.55	MRPL10 ^S	34.55	RPL37B ^{A, H}	28.79	RPS8A ^{A, C, P}	38.79	UBP3 ^{A, R}	37.35	YLR107W ^{A, S}	31.85
DAN1 ^S	31.28	MRPL39 ^A	42.77	RPL43A ^A	25.87	RPS9B ^{A, C, H, P, R}	58.15	UME6 ^A	59.86	YLR149C ^R	48.28
DST1 ^A	41.95	MSL1 ^A	36.48	RPL6B ^S	32.80	RSA1 ^{A, C}	28.50	VPS35 ^A	66.70	YMR125W ^{A, P}	22.40
DST1 ^C	63.51	NUP2 ^S	52.80	RPL9A ^{A, C}	28.73	RSM23 ^{A, H}	33.85	VPS70 ^{H, R}	50.56	YMR190C ^P	74.51
ECM32 ^A	63.86	OAZ1 ^R	27.51	RPS0A ^A	41.02	SAC3 ^{A, C, R}	37.40	XRN1 ^{A, C}	56.29	YNL171C ^A	53.33
EFM2 ^A	37.93	PHO80 ^R	27.40	RPS16A ^{C, P}	28.69	SHE4 ^R	24.23	YAK1 ^A	34.74	YNR048W ^R	30.03
EFT2 ^A	50.11	PRC1 ^{A, H, R, S}	30.49	RPS17A ^{A, H, S}	41.72	SIL1 ^A	32.76	YAL029C ^H	33.76	YOR302W ^S	40.77
GAS2 ^R	45.70	RBG2 ^A	31.61	RPS18B ^S	33.71	SLX9 ^A	51.39	YAR1 ^A	47.35	YPL069C ^C	25.92
GCN3 ^A	23.77	REI1 ^A	46.34	RPS19A ^{A, S}	31.38	STB3 ^A	52.56	YBR064W ^{A, S}	63.15	YPL086C ^R	29.68
GIS2 ^A	55.83	RKM3 ^A	44.84	RPS1B ^A	25.97	STM1 ^R	56.00	YCL009C ^A	26.78	YPL197C ^{A, S}	33.40
GLC3 ^A	23.76	RKM4 ^A	31.27	RPS21A ^A	30.87	SUC2 ^H	43.56	YDL116W ^{A, S}	35.84		
GLG2 ^S	55.03	RPA34 ^R	33.84	RPS23B ^A	32.85	TAE1 ^A	27.61	YEL009C ^{A, P}	27.71		
GSY1 ^{P, S}	32.14	RPL12B ^{P, R}	23.11	RPS24B ^P	37.13	TEF4 ^S	51.02	YER081W ^S	48.85		

More importantly, when grown on rapamycin-containing media, this gene was shown to be associated with several members of the mTOR pathway: *TOR1* (39.02% reduction, rapamycin, acetic acid) and *STM1* (45.28% reduction, rapamycin) ($p=3.33E-01$) activated during starvation conditions and inhibited by rapamycin treatment (Fig. 3.6; Table 3.6). Interestingly, GO analysis showed co-expression pattern between *STM1* and *HSC82* (35.91% reduction, rapamycin), as well as previously described genetic interactions between *HSC82* and *TOR1* (Fig. 3.6, 3.7, Table 3.6).⁶⁴ The greatest growth reduction in *DOM34Δ* SGA was exhibited by gene deletions responsible for various ribosomal functions (Table 3.6). Interesting exception included *BSC1* (YDL037C; 66.95% reduction; acetic acid), a protein of unknown function which has a similarity to a family of proteins required for invasive growth and biofilm formation (Table 3.6). Another gene deletion of which resulted in great growth inhibition was *GSY1* (68.23% reduction, paromomycin) (Table 3.6). This gene codes for a glycogen synthase and has been implicated in response to various types of stress. The latter interaction confers great potential for *DOM34* to be involved in IRES, since it links the gene to stress response.

3.6.4 Gene ontology analysis of *MRPS16Δ* genetic interaction network

SGA screen for candidate *MRPS16Δ* associated the gene with structural components and regulatory proteins of both large and small cytosolic ribosomal subunits (Tables 3.3, 3.4). As a further link to translation, *MRPS16Δ* was found to genetically interact with *ANB1* (41.67% reduction, SGA & paromomycin) gene encoding an eIF5A protein, having a function in both initiation and elongation steps of protein synthesis. Greatest growth reduction in SGA occurred in combination of *MRPS16Δ* with ribosomal genes as well as the cap-dependent translation inhibitor *EAP1Δ* (71.02% reduction; paromomycin) (Table 3.7). More unusual is a decrease in colony size when query strain was combined with *TOS6Δ* (68.92% reduction; acetic acid)

Figure 3.6. Interaction network for *DOM34Δ* rapamycin-conditioned SGA. Double deletions (*DOM34Δ* x translation array) were grown on rapamycin-containing media. Dark grey query genes are those identified in the screen to have genetic interactions with *DOM34* revealed through diminished colony growth. In pale grey are highlighted genes with the strongest interaction (physical, genetic, co-expression, etc.) to the query translation-relevant genes from the rest of the genome compiled by GeneMANIA. These aid in the identification of other mechanisms this network is linked to, potentially linking our candidate deletion to important processes outside translation pathway. It can be seen from the number of additional (pale grey) genes that several processes were identified associate with this network, only those networks with the lowest false discovery rate (probability that an interaction is a false positive) were considered in the study (for full summary of these, see Tables 6,7). In this network, we focused on the TOR signaling cascade. It is an important mechanisms for attenuating canonical translation during the starvation stress response, therefore an important association for a potential ITAF element. Note that genetic interactions were found between *HSC82* and *TOR1*.

Table 3.6. SGA screen data of *DOM34Δ*. Strain collection was created combining *DOM34Δ* with deletions of translation-related genes. Colony size was scored after growing resultant strains on plain YPD at 30°C (S), on YPD with 37°C heat shock (H), in presence of acetic acid (A), cycloheximide (C), paromomycin(P) and rapamycin (R). Percent size reduction is indicated for each gene. (*) symbols indicate DryGin data collected via similar experiments by Dr. Boone from University of Toronto.¹²¹ The greater is the reduction in colony size, the stronger genetic interactions between the two deleted genes are. Of particular interest were interacting partners showing %size decrease of above 70%.

<i>DOM34Δ</i>							
AAH1 ^C	36.59	RPL21B ^P	48.04	SHE4 ^R	24.04	YMR31 ^{C,P}	62.35
AIM23 ^A	26.51	RPL22A ^A	47.56	SLX9 ^{A,P}	57.99	YNL087W ^P	50.52
ARX1 ^{A,P}	34.71	RPL23A ^{A,C,P}	52.15	STF1 ^A	27.06	YNL269W ^{A,R}	29.51
BRR1 ^{A,R}	54.88	RPL24A ^{A,C,P}	35.46	STM1 ^R	45.28	YNL277W ^P	23.90
BUD23 ^P	56.93	RPL24B ^C	40.39	SYS1 [*]	39.67	YOR017W ^C	44.90
CDH1 ^{A,S}	34.79	RPL34B ^P	49.60	TAE1 ^A	31.44	YOR028C ^A	22.96
CYC1 ^C	29.24	RPL36A ^C	52.16	TGS1 ^{A,C,S}	40.94	YOR302W ^{P,R}	50.23
DST1 ^P	39.08	RPL37B ^{A,C}	27.84	THI2 ^A	45.16	YPL048W ^P	44.24
EFT2 ^A	44.17	RPL9A ^{A,P}	62.26	TIF4632 ^C	61.65	ZDS1 ^C	49.23
GCN3 ^A	27.94	RPP1A ^P	30.14	TMA20 ^{*,P}	27.20		
GIS2 ^C	38.56	RPS0A ^P	37.19	TMA7 ^R	34.05		
GLC3 ^A	26.54	RPS0B ^C	41.48	TOR1 ^{A,R}	39.02		
GLG2 ^R	59.38	RPS10A ^A	49.00	TPK1 ^A	51.34		
GRX3 ^A	45.59	RPS10A ^{R,S,H}	26.76	TRM1 ^{A,R}	31.15		
GSY1 ^P	68.23	RPS11B ^A	46.87	TRP1 ^A	23.14		
GUF1 ^R	65.63	RPS14A ^P	66.90	TSA1 ^{C,R}	32.28		
HCR1 ^P	24.94	RPS16A ^{A,P}	56.24	UBP3 ^P	41.54		
HHF1 ^{A,C,P}	50.89	RPS17A ^P	37.22	UME6 ^P	33.93		
HHO1 ^A	49.30	RPS18B ^{A,P}	44.95	VMA3 [*]	42.11		
HPM1 ^P	44.10	RPS19A ^P	29.35	VPS35 ^{C,P}	35.87		
HSC82 ^R	35.91	RPS1A ^{*,A,C,P,R}	41.35	XRN1 ^{A,P}	48.66		
HSP30 ^A	33.38	RPS1B ^{*,P}	43.56	YAL012W ^P	66.41		
HTA1 ^R	29.92	RPS21A ^P	26.69	YAP1 ^{R,*,A,C,H}	23.91		
IMG2 ^{A,P}	27.47	RPS22B [*]	30.72	YBL079W ^{A,C}	48.23		
INO1 ^C	37.83	RPS23A [*]	35.51	YBR062C ^{A,P,R}	51.78		
LOC1 ^P	28.43	RPS24A ^P	28.14	YCL009C ^P	26.09		
LTV1 ^P	38.26	RPS24B ^{*,A}	33.85	YCL029C ^A	61.56		
MCK1 ^{C,R}	61.34	RPS26B ^A	31.44	YDL037C ^A	66.95		
MRPL1 ^A	61.51	RPS27B ^P	44.95	YDL130W ^A	41.59		
MRPL10 ^R	37.12	RPS28A [*]	44.91	YDR382W ^P	33.93		
MRPL9 ^R	33.52	RPS28B [*]	33.75	YDR515W ^P	25.02		
MRPS17 ^P	41.46	RPS29A [*]	34.11	YDR535C ^{A,R}	29.62		
NOP12 ^P	35.59	RPS29B ^{*,P}	52.21	YEL048C ^C	29.76		
NOP16 ^P	69.82	RPS30A [*]	58.72	YER086W ^P	29.93		
NTH1 ^{A,R}	29.39	RPS30B [*]	35.98	YJL051W ^P	27.78		
OAZ1 ^C	56.07	RPS4A ^A	32.56	YJL056C ^{A,C}	26.98		
PBP1 ^A	43.54	RPS4B ^{*,P}	42.60	YJL124C ^A	37.40		
PCH2 ^P	23.68	RPS6A [*]	36.25	YJL152W ^C	24.73		
PCI8 ^{A,C}	26.34	RPS6B [*]	58.55	YJL176C ^A	47.25		
PET54 ^C	30.77	RPS7A ^P	28.37	YJR003C [*]	65.64		
PHO80 ^R	38.08	RPS7B ^C	23.20	YJR137C ^{A,P}	31.93		
PRC1 ^A	39.60	RPS8A ^{A,P}	29.89	YKL074C ^P	31.29		
RBG2 ^A	32.10	RPS9B ^P	37.29	YKL205W ^A	38.00		
REI1 ^P	36.02	RSA1 ^P	28.31	YLR107W ^A	25.70		
RMD1 ^A	29.24	RSM25 ^A	50.66	YLR434C [*]	23.84		
RPA14 ^{A,P}	73.55	RSM27 ^A	45.61	YLR451W ^A	41.65		
RPA34 ^{A,S}	48.99	SAC3 ^P	40.70	YMR125W ^P	37.98		
RPL12B ^P	67.98	SBP1 ^A	43.80	YMR171C ^{A,R}	46.43		
RPL13A ^P	36.01	SDA1 [*]	30.03	YMR190C ^{A,C}	23.17		
RPL16B ^P	37.27	SER33 ^C	33.66	YMR307W ^C	32.50

Figure 3.7. Interaction network for *DOM34Δ* rapamycin-conditioned SGA. Double deletions (*DOM34Δ* x translation array) were grown on rapamycin-containing media. Dark grey query genes are those identified in the screen to have genetic interactions with *DOM34* revealed through diminished colony growth. In pale grey are highlighted genes with the strongest interaction (physical, genetic, co-expression, etc.) to the query translation-relevant genes from the rest of the genome compiled by GeneMANIA. These aid in the identification of other mechanisms this network is linked to, potentially linking our candidate deletion to important processes outside translation pathway. It can be seen from the number of additional (pale grey) genes that several processes were identified associate with this network, only those networks with the lowest false discovery rate (probability that an interaction is a false positive) were considered in the study (for full summary of these, see Tables 6,7). In this network, we focused on the TOR signaling cascade. It is an important mechanisms for attenuating canonical translation during the starvation stress response, therefore an important association for a potential ITAF element. Note the co-expression patterns of HSC82, TOR1 and STM1.

Table 3.7. SGA screen data of *MRPS16Δ*. Strain collection was created combining *MRPS16Δ* with deletions of translation-related genes. Colony size was scored after growing resultant strains on plain YPD at 30°C (S), on YPD with 37°C heat shock (H), in presence of acetic acid (A), cycloheximide (C), paromomycin(P) and rapamycin (R). Percent size reduction is indicated for each gene. (*) symbols indicate DryGin data collected via similar experiments by Dr. Boone from University of Toronto.¹²¹ The greater is the reduction in colony size, the stronger genetic interactions between the two deleted genes are. Of particular interest were interacting partners showing %size decrease of above 70%.

<i>MRPS16Δ</i>			
AEP2 ^S	54.94	RPS24B ^P	68.77
ANB1 ^{P, S}	41.67	RPS26B ^R	41.34
ATP22 ^A	24.31	RPS27B ^P	38.76
BRR1 ^R	45.94	RPS4B ^P	53.46
BUD21 ^P	37.77	RPS8A ^P	34.87
BUD27 ^A	60.19	RPS9B ^P	28.35
CDH1 ^{P, S}	44.39	RSA1 ^P	42.65
CIS3 ^R	56.11	SAC3 ^A	51.10
CLN3 ^A	51.80	SCD6 ^A	59.57
DST1 ^R	26.31	STF1 ^A	37.73
EAP1 ^P	71.02	SWS2 ^S	43.89
ECM1 ^R	26.33	TEF4 ^A	41.73
EDC2 ^A	32.56	TIF1 ^R	59.16
EFM2 ^A	58.67	TMA20 ^{A, P}	50.01
GLC3 ^{C, P}	36.73	TOS6 ^A	68.92
GLC3 ^P	31.70	UBP3 ^P	50.79
GUF1 ^R	48.13	UME6 ^A	57.67
HHT1 ^{P, R, A}	40.93	VPS35 ^R	48.04
HPM1 ^S	39.56	WHI3 ^{A, S}	47.99
NTH1 ^S	56.00	WHI5 ^{A, P, R}	27.96
RBG2 ^S	52.28	YAK1 ^A	49.43
RKM2 ^{P, S}	39.23	YAL012W ^A	59.24
RPL12B ^A	35.55	YAR1 ^A	54.29
RPL16A ^R	65.42	YBR064W ^A	43.66
RPL24A ^P	45.68	YBR089C-A ^S	27.41
RPL34A ^R	64.28	YDR382W ^{A, C, P}	58.77
RPL37B ^P	44.25	YGL035C ^A	36.88
RPL41A ^R	56.85	YGR285C ^P	27.22
RPL42A ^R	29.92	YJL080C ^S	29.94
RPL6B ^A	43.20	YJL089W ^{A, C, S}	45.35
RPL8B ^P	55.96	YJL160C ^R	44.42
RPS10A ^{A, R}	35.39	YJL176C ^{P, R}	44.46
RPS11B ^A	79.00	YKR006C ^P	30.56
RPS17A ^P	34.20	YLR085C ^S	37.30
RPS23B ^R	28.60	YOL115W ^P	38.81

(Table 3.7). This gene codes for a cell wall component expression of which varies with environmental perturbations and lack of which increases lactic acid resistance. Another interesting interaction is an association of MRPS16 with processes governing nucleosomal remodeling (*ARP6*, *NHP6B*, *BDF1*, *SWC5*) ($p=1.05E-02$) during changes in cellular conditions (Fig. 3.8). Note that *ARP6* has both genetic and physical interactions with vacuolar genes (*VPS71* and *VPS74*)(Fig.3.8). Strong association of this network with stress response can help in implication of the candidate in IRES-mediated translation.

3.6.5 Gene ontology analysis of *RPL36AΔ* genetic interaction network

RPL36A is a structural constituent of a large ribosomal subunit. Therefore, its affiliation with various other ribosomal and translation-related genes in the identified genetic interaction network is not surprising (Table 3.3, 3.4). However, when noting the greatest growth reductions, some interesting interactions were discovered. One of these, *PDR1*(*YGL013C*; 82.50% reduction; paromomycin) is involved in regulation of the multidrug resistance genes (Table 3.8). Another, *SWI3* (72.22% growth reduction; acetic acid), is a chromatin remodeling complex, necessary for transcription of many genes including *GCN4*, an activator of amino acid biosynthesis during starvation (Table 3.8). As well, gene *YDR282CΔ* has shown substantial growth reduction in combination with *RPL36AΔ* (74.05%; acetic acid) (Table 3.8). No function was confirmed for the protein product of this gene. However, it was identified in the original screen for gene deletions affecting IRES expression (Table 3.2) though not selected for follow-up. Curiously, through different treatments of conditional SGA, it has been implicated in various other processes. For instance, general SGA screen showed association of *RPL36A* with serine hydrolase within the vacuole *PRC1* (55.94% reduction, SGA & acetic acid) ($p=1.01E-02$). In presence of rapamycin, serine family amino acid metabolism was linked to *RPL36AΔ* ($p=9.14E-03$)(Fig. 3.9).

Figure 3.8. Interaction network for *MRPS16Δ* general SGA. Double deletions (*MRPS16Δ* × translation array) were grown in optimal conditions. Dark grey query genes are those identified in the screen to have genetic interactions with *MRPS16* revealed through diminished colony growth. In pale grey are highlighted genes with the strongest interaction (physical, genetic, co-expression, etc.) to the query translation-relevant genes from the rest of the genome compiled by GeneMANIA. These aid in the identification of other mechanisms this network is linked to, potentially linking our candidate deletion to important processes outside translation pathway. It can be seen from the number of additional (pale grey) genes that several processes were identified associate with this network, only those networks with the lowest false discovery rate (probability that an interaction is a false positive) were considered in the study (for full summary of these, see Tables 6,7). In this network, we focused on the nucleosome organization, as an unusual process to be associated with a mitochondrial gene. Nevertheless, it is an important regulatory process affected during stress response, therefore should be noted when evaluating the potential role of *MRPS16Δ* as an ITAF element.

Table 3.8. SGA screen data of *RPL36A2Δ*. Strain collection was created combining *RPL36AΔ* with deletions of translation-related genes. Colony size was scored after growing resultant strains on plain YPD at 30°C (S), on YPD with 37°C heat shock (H), in presence of acetic acid (A), cycloheximide (C), paromomycin(P) and rapamycin (R). Percent size reduction is indicated for each gene. (*) symbols indicate DryGin data collected via similar experiments by Dr. Boone from University of Toronto.¹²¹ The greater is the reduction in colony size, the stronger genetic interactions between the two deleted genes are. Of particular interest were interacting partners showing %size decrease of above 70%.

<i>RPL36AΔ</i>							
AAP1 ^S	37.26	RML2 ^P	33.66	SIL1 ^A	49.46	YJL089W ^R	44.46
APE2 [*]	44.24	RPA14 ^A	41.87	SIP2 ^P	48.47	YJL095W ^R	33.78
ARD1 [*]	46.55	RPL12B ^P	26.16	SLX9 ^P	57.78	YJL124C ^A	54.21
ARX1 ^P	39.23	RPL13A ^A	57.30	SSB1 ^P	43.57	YJL176C ^A	72.22
ASC1 ^P	37.32	RPL16B ^P	32.18	SSF2 ^R	53.11	YKL205W ^A	33.92
BUD23 ^P	27.73	RPL19A ^{C,P}	58.45	STB3 ^A	48.02	YLR050C [*]	40.11
CDH1 ^S	53.09	RPL20B ^S	58.50	STF1 ^A	57.37	YLR451W ^P	26.50
CLN3 ^P	60.05	RPL22A ^P	45.22	TAE2 ^R	39.38	YMR125W ^P	32.57
COG3-1 [*]	32.78	RPL22B ^P	26.20	THP2 [*]	31.11	YMR172W ^P	36.24
COX23 [*]	25.28	RPL24A ^A	40.96	TMA19 [*]	49.56	YMR190C ^P	35.97
DOA1 [*]	45.51	RPL27A ^P	51.77	TMA64 ^P	25.38	YMR194C-A ^P	48.70
DPH2 ^P	55.65	RPL27B ^A	50.85	TOM1 [*]	41.12	YMR31 ^A	45.12
DST1 ^{P,S}	54.26	RPL29 ^P	25.67	TRM1 ^P	42.16	YOR115C ^P	25.89
EAP1 ^{A,C,P,R}	44.99	RPL2A ^P	36.34	UBC4 [*]	60.97	YOR302W ^P	54.03
ECM1 ^P	22.77	RPL34A ^S	34.07	UBP3 ^{P,*}	40.86	YPL086C ^R	57.75
EDC2 ^P	26.06	RPL35B ^A	87.79	UTR2 [*]	28.23	YPL183W-A ^P	37.70
ERG11 [*]	51.91	RPL37B ^A	23.12	VPS27 [*]	21.79		
GLC3 ^P	29.73	RPL43A ^P	34.70	VPS29 [*]	26.84		
GSY1 ^{A,R}	27.97	RPL6B ^{A,H,P,R}	36.07	WHI3 ^{A,P,R}	44.64		
HHT1 ^{A,C,P}	37.53	RPN11 [*]	57.45	WHI5 ^{A,S}	42.88		
HPM1 ^P	31.08	RPP1A ^P	34.96	XRN1 ^P	52.03		
HTZ1 ^P	43.58	RPS0A ^P	39.70	YAL012W ^P	53.13		
INO1 ^R	53.42	RPS0B ^A	47.74	YAL029C ^A	36.48		
ISW2 [*]	25.64	RPS10A ^A	49.34	YBR057C ^A	32.75		
JNM1 [*]	37.39	RPS16A ^P	50.26	YDL116W ^P	26.32		
LOC1 ^P	33.48	RPS17A ^A	28.67	YDR282C ^A	74.05		
LTV1 ^P	29.23	RPS18A ^P	28.35	YDR382W ^P	34.10		
MRP49 ^P	44.21	RPS19B ^A	66.35	YDR515W ^P	27.44		
MRPL39 ^P	23.29	RPS1A ^A	53.78	YEL020C [*]	38.57		
NTH1 ^{A,P}	24.15	RPS1B ^P	50.68	YER086W ^P	38.72		
NUP2 ^P	39.83	RPS27B ^A	48.36	YER088C ^P	64.42		
PAT1 [*]	25.13	RPS29B ^S	30.13	YFL031W ^P	49.96		
PCH2 ^P	44.75	RPS30B ^P	59.83	YGL013C ^P	82.50		
PCI8 [*]	32.14	RPS4B ^A	33.89	YGL035C ^P	26.97		
PRC1 ^{A,S}	55.94	RPT2 [*]	48.58	YGR053C ^P	32.38		
PXA2 [*]	58.76	RSA1 ^P	46.50	YGR285C ^{A,C,H,P,R}	53.18		
RAD61 [*]	40.13	SAC3 ^P	35.17	YHR121W ^A	26.06		
REI1 ^P	30.24	SAF1 ^P	32.90	YJL051W ^A	38.52		
RIC1 ^P	85.05	SEC17 [*]	81.02	YJL056C ^P	41.43		
RMD1 ^A	32.19	SHF1 [*]	54.33	YJL080C ^A	45.05

Figure 3.9. Interaction network for *RPL36AΔ* rapamycin-conditioned SGA. Double deletions (*RPL36AΔ* x translation array) were grown on media containing rapamycin. Dark grey query genes are those identified in the screen to have genetic interactions with *RPL36A* revealed through diminished colony growth. In pale grey are highlighted genes with the strongest interaction (physical, genetic, co-expression, etc.) to the query translation-relevant genes from the rest of the genome compiled by GeneMANIA. These aid in the identification of other mechanisms this network is linked to, potentially linking our candidate deletion to important processes outside translation pathway. It can be seen from the number of additional (pale grey) genes that several processes were identified associate with this network, only those networks with the lowest false discovery rate (probability that an interaction is a false positive) were considered in the study (for full summary of these, see Tables 6,7). In this network, we highlighted the serine amino acid metabolic process, since it can be influenced by starvation conditions, providing a link between *RPL36AΔ* and stress response that is logical for a protein involved in IRES-mediated translation. Note genetic and physical interactions exhibited by *TAE2*, a gene involved in response to stalled translation response. Involvement of this interacting partner in many processes may be a good support for *RPL36A* having a role of an ITAF.

In presence of acetic-acid, it demonstrated genetic interactions with LSM1 (54.21% reduction, acetic acid), which is part of the complex involved in mRNA catabolism ($p=1.31E-02$). When under influence of translation elongation-blocking cycloheximide, *RPL36AΔ* has been linked to *ZUO1* (53.18% size reduction; acetic acid, cycloheximide, heat shock, paromomycin & rapamycin), a protein involved in both ribosomal biogenesis and proper folding of peptides ($1.52E-02$). As well, it displays genetic interactions with chromatin-remodeling gene *HHT1* (37.53% reduction, acetic acid, cycloheximide & rapamycin).

An interesting result arose from the rapamycin-conditioned SGA. *TAE2* (39.38% reduction, rapamycin) has been identified, a protein of ribosome quality control complex (RCQ).⁶⁵ GO analysis identified both physical and genetic interactions of *TAE2* with cytoplasmic and mitochondrial ribosomal proteins as well as some of the transcription factors regulating those (Fig.3.9). Interestingly, it was later found that *TAE2* was co-expressed with only 2 of these (*ELP2*, *ZUO1*, data not shown). In general, *RPL36A* interaction network exhibited a variety of processes, some of which logically link it to stress response. The multitude of ribosomal factors picked up validates the screen, since the candidate is a ribosomal constituent.

3.6.6 Gene ontology analysis of *VPS70Δ* genetic interaction network

VPS70, a protein with a vacuolar function, exhibited a surprising number of genetic interactions with components of the ribosome and translation regulatory elements. For instance, component of a large ribosomal subunit *RPL9A* demonstrated 85.45% growth reduction in acetic acid, and member of a small ribosomal subunit *RPS9B* exhibited 81.83% growth reduction in media containing acetic acid and rapamycin (Table 3.9). In addition to those interactions, *VPS70Δ* showed genetic interactions with meiosis regulator *UME6* (80.17% reduction, SGA & acetic acid) and, alike to *ATP22Δ*, a component of nuclear pore complex *NUP84*, (69.85% reduction, SGA & heat shock) (Table 3.9). Under optimal conditions, it was also found to have genetic

Table 3.9. SGA screen data of *VPS70Δ* & *YBR064WΔ*. Strain collection was created combining *VPS70Δ* & *YBR064WΔ* with deletions of translation-related genes. Colony size was scored after growing resultant strains on plain YPD at 30°C (S), on YPD with 37°C heat shock (H), in presence of acetic acid (A), cycloheximide (C), paromomycin(P) and rapamycin (R). Percent size reduction is indicated for each gene. (*) symbols indicate DryGin data collected via similar experiments by Dr. Boone from University of Toronto.¹²¹ The greater is the reduction in colony size, the stronger genetic interactions between the two deleted genes are. Of particular interest were interacting partners showing %size decrease of above 70%.

VPS70				YBR064W			
AAH1 ^P	44.35	RPS8A ^A	35.46	BRR1 ^C	27.30	RPS22A ^A	25.33
ASC1 ^R	24.19	RPS9B ^{A,R}	81.83	BUD13 [*]	56.80	RPS27B ^{A,P}	27.65
BOR1 [*]	40.04	RSA1 ^P	78.25	BUD21 ^A	43.89	RPS7A ^S	68.54
BUD22 ^A	39.39	SAC3 ^A	30.99	CDC3 [*]	48.72	RPS9B ^{A,S,C}	45.83
BUD27 ^S	46.65	SLM4 [*]	37.48	CLN3 ^S	65.73	RSA1 ^P	54.67
CLU1 ^R	46.83	STM1 ^R	51.50	DGK1 [*]	29.79	RSM25 ^R	44.61
DDC1 [*]	32.55	TAE1 ^R	45.23	DST1 ^A	36.48	SAC3 ^A	70.36
DOM34 ^R	30.91	TOR1 ^R	29.67	EFM2 ^S	38.56	SLU7 [*]	51.54
DST1 ^R	58.81	TPK1 ^A	60.36	EFT1 ^S	39.40	SNT309 [*]	40.49
EAP1 ^{A,C,R}	54.08	TRM1 ^R	23.43	EFT2 ^S	49.80	SNU114 [*]	25.60
EAP1 ^R	47.37	TSA1 ^R	62.67	ETR1 ^A	53.67	STF1 ^A	56.38
erg11 [*]	36.28	UBI4 [*]	68.30	HSC82 ^A	63.41	TAE1 ^S	33.81
GBP2 ^{A,S}	41.99	UME6 ^{A,S}	80.17	IST3 [*]	67.75	TMA20 ^A	38.23
GCN3 ^A	47.28	VMA9 [*]	27.06	LEA1 [*]	60.83	TOR1 ^R	53.34
GIS2 ^{A,P}	45.68	VPS35 ^R	50.25	LOC1 ^S	58.71	VPS70 ^{A,R,S}	49.18
GSY2 ^R	27.79	WHI5 ^{A,R}	52.29	MET17 ^A	49.35	WHI3 ^R	23.75
HHF1 ^A	49.62	XRN1 ^{R,S}	57.61	NOP12 ^P	57.57	WHI5 ^{A,S}	35.26
HHT1 ^S	31.59	YAK1 ^A	36.02	NOP16 ^A	90.48	XRN1 ^S	41.74
IES2 [*]	73.09	YAL012W ^A	27.51	NTH1 ^A	41.22	YAK1 ^A	39.20
KES1 [*]	54.29	YAR018C ^S	35.66	PCH2 ^R	64.39	YBL079W ^S	25.91
LOC1 ^S	26.50	YCL009C ^A	50.06	PRP18 [*]	26.24	YBR062C ^A	35.35
LTV1 ^A	41.39	YDL116W ^{H,S}	69.85	PRP22 [*]	65.97	YBR064W ^S	38.37
MCK1 ^R	39.24	YDL130W ^A	35.86	REI1 ^S	57.98	YCL009C ^A	75.90
MRP49 ^A	47.77	YDR382W ^A	23.23	RKM2 ^A	62.89	YDR378C ^A	35.63
PHO80 ^A	42.08	YEL009C ^{P,S}	30.73	RKM3 ^A	24.11	YEL009C ^P	34.59
PRC1 ^{A,C,R}	46.09	YEL020C [*]	50.18	RPL20B ^A	58.11	YER086W ^A	31.05
PUB1 [*]	60.22	YER086W ^A	39.48	RPL23A ^S	76.17	YGL151W ^S	27.57
RBG2 ^A	33.79	YFL034W ^S	40.13	RPL24A ^S	40.08	YGL162W ^R	37.12
RKM2 ^S	38.19	YGL035C ^A	62.01	RPL2B ^{A,C}	51.46	YHC1 [*]	36.34
RPA14 ^R	46.60	YGL162W ^S	34.66	RPL34B ^A	27.53	YJL051W ^S	35.35
RPA34 ^{A,R}	49.82	YGR276C ^A	61.04	RPL43A ^S	30.36	YKR020W ^S	31.25
RPL12B ^{A,P}	62.75	YJL051W ^P	32.04	RPL6B ^A	39.85	YLR085C ^S	30.61
RPL27A ^S	33.36	YJL056C ^A	25.54	RPL9A ^A	43.64	YLR398C ^R	36.89
RPL34B ^R	45.98	YJL080C ^A	41.18	RPP1A ^S	39.41	YLR451W ^A	36.54
RPL6B ^A	28.00	YJL124C ^P	47.59	RPS0A ^{C,P}	42.59	YMR125W ^A	57.61
RPL9A ^A	85.45	YLR085C ^S	26.53	RPS10A ^{A,R}	30.87	YMR179W ^R	63.45
RPP1A ^R	42.51	YMR125W ^A	23.13	RPS11B ^S	45.22	YMR302C ^A	36.89
RPS10A ^S	24.99	YNL269W ^A	44.70	RPS17A ^{C,S}	73.12	YOL006C ^S	50.46
RPS16A ^R	30.72	YOL115W ^A	44.50	RPS19B ^A	60.05	YOL115W ^A	77.05
RPS17A ^A	38.81	YOR302W ^A	28.65	RPS1B ^{C,P}	62.72	YPL069C ^A	31.80
RPS19B ^A	42.51	YPL086C ^S	31.16	RPS21A ^P	43.15	YPL086C ^R	37.02
RPS24B ^A	47.71	YPT6 [*]	31.84				
RPS27B ^A	42.60	YSR3 [*]	50.36				
RPS7A ^R	59.49	ZDS1 ^R	40.16				

interactions with *ARP6* (26.53% reduction, SGA), member of the histone remodeling complex Swr1 ($p=1.38E-03$) (Table 3.3). In general, the association between vacuolar (therefore stress-related) protein and the ribosomal proteins in the interaction network is favorable, as it may help in implication of *VPS70* in IRES-mediated translation.

3.6.7 Gene ontology analysis of *YBR064W*Δ genetic interaction network

Despite having an unknown function, *YBR064W* exhibited numerous genetic interactions with ribosomal proteins. Interestingly, those are mostly associated with each other by co-expression patterns as opposed to physical or genetic interactions (Fig.3.10). Noting those strains that showed the greatest size reduction, *YBR064W* appears to be associated with various stages of ribosomal biogenesis. NOP16, for instance, is a constituent of the pre-ribosomal particles (90.48% reduction, acetic acid); PAP2 is involved in post-transcriptional modification (specifically polyadenylation) of rRNA precursors (77.05% reduction; acetic acid), and SAC3 is a member of a nucleopore complex necessary for biogenesis of the 40S subunit (70.36% reduction; acetic acid) (Table 3.9). As well, RPL23A (76.17% reduction, SGA) and RPS17A (73.12% reduction, SGA & cycloheximide) are structural components of the large and small ribosomal subunits, respectively (Table 3.9). Other enriched proteins include cyclin-encoding *CLN3* (65.73% reduction, SGA) and histone-remodeling *ARP6* (30.61% reduction, SGA), which were discovered to have genetic interactions with *YBR064W* in the general SGA analysis (Fig. 3.10). Finally, a component of a branched-chain amino acid biosynthesis *ILV6*Δ(*YCL009C* Δ) demonstrated a substantial decrease in cell growth when combined with *YBR064W*Δ (75.90% reduction; acetic acid) (Table 3.9). Overall, this candidate exhibited numerous interactions with a variety of proteins that may complicate the establishment of its role as an ITAF.

Figure 3.10. Interaction network for *YBR064WΔ* general SGA. Double deletions (*YBR064WΔ* × translation array) were grown in optimal conditions. Dark grey query genes are those identified in the screen to have genetic interactions with *YBR064W* revealed through diminished colony growth. In pale grey are highlighted genes with the strongest interaction (physical, genetic, co-expression, etc.) to the query translation-relevant genes from the rest of the genome compiled by GeneMANIA. These aid in the identification of other mechanisms this network is linked to, potentially linking our candidate deletion to important processes outside translation pathway. It can be seen from the number of additional (pale grey) genes that several processes were identified associate with this network, only those networks with the lowest false discovery rate (probability that an interaction is a false positive) were considered in the study (for full summary of these, see Tables 6,7). In this network, note the large amount of different kinds of interactions. Most genes are interconnected by co-expression (for a gene with unknown function, not surprising), fewer by genetic interactions, the smallest number are shown to interact physically. Note also the abundance of ribosomal components (pale grey genes identified by RPS and RPL). The latter observation may indicate good support for *YBR064W* having a role of an ITAF.

3.7 Analysis of candidates' genetic interactions identified through SDL

In an analogous manner to double gene deletion analysis, the effect of gain-of-function mutations can be evaluated. Previous studies characterized only ~15% of the yeast genes as toxic in an over-expressed state. These were mostly transcription factors and signaling proteins involved in transport, secretion, cellular organization and cell cycle control.⁶⁶ Proteins governing regulatory functions in the cells are normally manufactured in precise quantities to maintain proper stoichiometric ratios with interacting components.⁵⁵ As well, they are expressed specific to particular stages in the cell cycle. Temporal deregulation as well as an increase in protein abundance that are characteristic of the constitutive overexpression engender the sick phenotype.⁵⁵

As a result of these studies, overexpression set analogous to the deletion array of approximately 5,000 genes was created to study genetic interactions.^{54,55} If effect of deletion of gene Y and overexpression of gene Z in the same cell population cannot be inferred from their separate phenotypes, the genes are likely interacting genetically. SGA methodology has been adapted, employing a set of plasmids over-expressing a particular gene.⁵⁵ The idea is that overexpression of a protein will not cause an adverse effect in a healthy cell population, but combined with a deletion of an interacting partner will result in a quantifiable phenotype.⁵⁵ In this manner it is thought that the imbalance between interacting partners may be lethal to the cell.

We tested the effect of over-expressing our candidates in the translation deletion array to augment their genetic interaction networks. Only a few interesting patterns emerged, however. *RPL36AΔ* was found to have some associations with genes involved in translation regulation ($p=2.45E-03$), such as *SBP1* (Table 3.10). The gene product is a repressor of translation through eIF4G binding and has been known to bind mRNA 5'-UTR upon glucose starvation.⁶⁷ The

Table 3.10. Genes identified through the SDL screen (% size reduction not shown). *RPL36AΔ*, encoding a ribosomal particle, had the greatest number of interactions in the translation array.

<i>ATP22</i>	<i>DOM34</i>	<i>RPL36A</i>		<i>VPS70</i>	<i>YBR064W</i>
YOL115W	RPS24B	AIM23	RPS1B	HTZ1	BUD23
		BUD23	RSA1	YFL031W	ETR1
		HCR1	SBP1	YGL151W	LOC1
		HPM1	XRN1	YPL086C	RPL8B
		LTV1	YEL009C		YGR285C
		RPL34B	YER086W		
		RPL8B			

remaining interactions were mostly reiterating the results of our SGA screen, confirming previously identified data.

3.8 Analysis of candidates' genetic interactions identified through PSA

In an attempt to cover all genetic interactions exhibited by our candidates, we performed the phenotypic suppression analysis (PSA). As mentioned previously, single gene deletion may only exhibit an unhealthy phenotype during stressful conditions. One way to establish an interaction network of a product encoded by that gene is to identify the factors overexpression of which rescues the sick phenotype exhibited by that gene deletion.⁴⁵ Similarly to SGA and SDL analyses, an interacting gene likely implies involvement in the same pathway.⁴⁵

Additionally, a comparison of a strain harboring double deletion from an SGA study to a strain in PSA exhibiting deletion of one of those genes and overexpression of another, while subjected to the same stress, may confirm the phenotypic effect arising from deletion of the two genes.

By introducing a gene initially removed from a double deletion strain in SGA via an over-expression vector in PSA, it is possible to verify the phenotype created by the synergistic effect of the two deletions.

We were determining which over-expressed candidates would be able to rescue sick phenotypes of the translation deletion array when the latter is subjected to hostile environment.

A vector with an over-expressed ORF of each of the six candidates was introduced into the translation array to identify genomic deletions relevant to translation with sick phenotype, that could be compensated by the presence of a candidate gene. For all of the candidates, gene ontology analysis of their interactors demonstrated an enrichment of genes involved in regulation of translation, regardless of treatments (Table 3.11).

Comparable to the SGA analysis, this screen demonstrated numerous interactions of the candidates with ribosomal proteins and RNA, canonical elements of translation initiation and

Table 3.11. PSA analysis of the six candidates affecting IRES-mediated translation initiation. Single deletion strains were subjected to various stressors resulting in decreased fitness. Upon overexpression of a particular candidate gene, a sickness was suppressed, conferring the strain a healthy phenotype in the same conditions. Such associations between over-expressed and deleted genes are indicative of their genetic interactions with each other. Data for acetic acid, heat shock, cycloheximide, paromomycin, and rapamycin conditional screens are shown here. GO annotations are included for the enriched functions with the associated p-values where available. Note the enrichment of translation regulation, and structural constituents of ribosomal particles throughout.

	Acetic acid		Cycloheximide		Paromomycin		Rapamycin		Heat shock	
	GO	P-value	GO	P-value	GO	P-value	GO	P-value	GO	P-value
YBR064W	Structural constituent of ribosome	2.15E-05	Structural constituent of ribosome	3.53E-27	Mitochondrial large ribosomal subunit	2.44E-05	Cytoplasmic translation	1.62E-28	mRNA processing	3.37E-12
	RNA phosphodiester bond hydrolysis, endonucleolytic	1.15E-03	Regulation of translation	1.58E-10	structural constituent of ribosome	8.86E-05	Structural constituent of ribosome	2.01E-25	Regulation of translation	1.14E-06
VPS70	Structural constituent of ribosome	9.60E-14	Regulation of translation	7.44E-04	Regulation of translation	4.77E-26	Regulation of translation	1.23E-27	mRNA processing	2.24E-05
	Regulation of translation	6.78E-12	Structural constituent of ribosome	2.69E-03	Structural constituent of ribosome	9.65E-21	Structural constituent of ribosome	9.01E-17	-	-
RPL36A	rRNA metabolic process	1.36E-07	Structural constituent of ribosome	1.84E-07	Structural constituent of ribosome	1.24E-15	Structural constituent of ribosome	1.74E-19	mRNA processing	3.91E-10
	RNA phosphodiester bond hydrolysis, endonucleolytic	4.42E-05	rRNA metabolism	6.02E-06	Maturation of SSU-rRNA	8.81E-07	Regulation of translation	7.43E-14	Regulation of translation	2.11E-05
MRPS16	Structural constituent of ribosome	4.46E-07	Structural constituent of ribosome	2.62E-05	Regulation of translation	3.01E-18	Regulation of translation	2.29E-29	mRNA processing	5.94E-07
	Regulation of translation	9.09E-03	Regulation of translation	1.14E-03	rRNA metabolism	2.06E-17	Structural constituent of ribosome	4.63E-26	Regulation of translation	1.11E-03
DOM34	mRNA processing	3.51E-03	mRNA processing	2.80E-03	Structural constituent of ribosome	5.62E-15	Structural constituent of ribosome	1.36E-04	mRNA processing	8.57E-05
	-	-	rRNA metabolism	1.25E-03	rRNA metabolism	5.34E-05	mRNA processing	2.36E-03	-	-
ATP22	Regulation of translation	2.23E-14	Structural constituent of ribosome	2.74E-05	Regulation of translation	9.24E-19	Regulation of translation	1.61E-26	-	-
	rRNA metabolism	1.57E-06	Regulation of translation	2.23E-04	rRNA metabolism	3.69E-11	Structural constituent of ribosome	1.91E-19	-	-

elongation. Some proteins were picked up that are involved in the mitochondrial translation, but those interactions were less common than SGA screen results (*YBR064WΔ*, paromomycin screen, $p=2.44E-05$)(Table 3.11).

Of interest are those genes that were found through the SGA to cause a sick phenotype in combination with a candidate strain deletion, but resulted in a suppression of that phenotype when the candidate was introduced into a single deletion strain through an over-expressed vector in the same conditions. 28 of these genes were found in the screen (Table 3.12). Several of these were the ribosomal structural genes - *RPL12B*, *RPS16A*, *RPS17A*, *RPS18A*, *RPS1A* and *RPS9B*. Note that the latter was identified in combination with four candidates (*ATP22 Δ*, *DOM34 Δ*, *MRPS16 Δ* & *VPS70Δ*). Gene *ASC1*, a structural component of the 40S ribosomal subunit is also involved in replication stress and amino acid starvation (Table 3.12). Of proteins involved in the mitochondrial translation, only *MRP49* was found, a component of the large subunit, in a *VPS70Δ* acetic acid screens. Interestingly, *VPS70* itself was identified in the screens along with other vacuolar proteins *VPS35* and *PRC1* (Table 3.11). More specific to the stress response pathways, two heat-shock protein-encoding genes were identified, *HSP30Δ* and *HSC82Δ*, combined with *DOM34* and *YBR064W* gene deletions and over-expressed plasmids, respectively (Table 3.12). As well, a group of nutrient starvation - regulating genes was identified, including *GLC3* (glycogen storage), and *SIP4* (gluconeogenesis). In general, however, the results were similar to the SGA and SDL data, validating previously acquired results.

3.9 Re-introduction of candidate genes in their respective deletion strains to rescue sick phenotype

This study strongly relied on the phenotype exhibited by single and double deletion strains in different kinds of hostile environments. Precautions should be taken to make sure the measured effects are not due to extraneous factors present in the biological system, but are a direct result

Table 3.12. Overlap in the genes identified through SGA and PSA, specific to treatment. For instance, double deletion of *DOM34* and *RPS9B* on paromomycin resulted in no growth in SGA. A single deletion *RPS9B* strain also possesses a sick phenotype on paromomycin. Upon transformation with an over-expressed version of *DOM34*, the ill phenotype is suppressed, resultant strain shows growth. Although not a quantitative assay, this connection between the two data sets helps to validate results initially found in the SGA screen, affirming genetic interactions between the candidate and a deletion in question. This association builds a stronger case for the candidate to be involved in IRES-mediated translation if interactions confirmed in such a manner are relevant to stress response and translation pathways.

<i>ATP22 Δ</i>		<i>DOM34Δ</i>			<i>MRPS16Δ</i>		
<i>Paromomycin</i>	<i>Rapamycin</i>	<i>Acetic Acid</i>	<i>Paromomycin</i>	<i>Rapamycin</i>	<i>Acetic Acid</i>	<i>Paromomycin</i>	<i>Rapamycin</i>
RPS16A	GAS2	HSP30	BUD23	BRR1	TEF4	GLC3	VPS35
RPS9B	HAP4		RPS16A	RPS1A	YJL089W	RPS9B	
	RPL12B		RPS9B				
	VPS70						
<i>RPL36AΔ</i>		<i>VPS70Δ</i>		<i>YBR064WΔ</i>			
<i>Paromomycin</i>	<i>Rapamycin</i>	<i>Acetic Acid</i>	<i>Cycloheximide</i>	<i>Acetic Acid</i>	<i>Cycloheximide</i>		
ASC1	TAE2	MRP49	EAP1	HSC82	RPS17A		
RPS18A	YPL086C	PRC1					
		RPS9B					
		VPS70					

of gene dosage. To ensure this, we first transformed each of the single candidate deletions with a vector over-expressing the corresponding gene and an empty vector, then tested the resultant strains for growth on different antibiotics. If sick phenotype is a result of a gene deletion only, growth in stressful conditions should be re-established upon re-introduction of the gene but not an empty vector. Suppression of sickness by an empty vector would indicate the phenotype is due to effects unaccounted for and failure of the experimental system.

Re-introduction of the overexpression vectors into their respective deletion strains resulted in suppression of the sick phenotype in presence of the all of the tested chemicals. Spot test of *ATP22Δ*, *DOM3 Δ*, *MRPS16Δ*, *RPL36AΔ*, *VPS70Δ* and *YBR064WΔ* on acetic acid resulted in little or no growth when compared with a pure YPD control (Fig.3.11, left panels). The vector over-expressing each of the genes was transformed into the corresponding strains. The resultant cultures exhibited the amount of growth on acetic acid upon repeat of the spot test, that was comparable to their growth on a YPD control (Fig. 3.11, middle panels). Upon introduction of an empty plasmid not harboring an ORF into the same strains, no growth occurred on YPD with acetic acid (Fig. 3.11, right panels). Similar patterns were found in experiments on media containing paromomycin, cycloheximide, rapamycin and YPD grown at 37°C (heat shock) (data not shown).

Strains harboring double deletions (those of candidate gene with a deletion of its genetically interacting partner) should be subjected to a similar test. If an introduction of a vector with an over-expressed version of the candidate into such double-deletion strain rescues the sick phenotype when grown in presence of a chemical; while an empty vector yields no growth, the experimental system works in accordance with expectations. However, if transformation yields growth comparable to over-expression vector, then the phenotype is not influenced by the dosage of genes of interest but the factors unaccounted-for.

Figure 3.11. Candidate re-introduction spot test. Confirmation of acetic acid sensitivity as a result of a knock-out of one of the six candidates. Single deletion of a candidate gene produces no growth (left panels). Upon re-introduction of the vector over-expressing the candidate gene, strain exhibits phenotype comparable to wildtype (middle panels). Upon introduction of the empty vector (no ORF) into the strain no growth results (right panels). Experiment shows that the chemical sensitivity phenotype observed throughout the SGA, SDL and PSA experiments was specifically due to an absence of the candidate gene from a strain under study and not any extraneous effects on the system.

Re-introduction of the candidate genes into the double deletion strains yielded similar results (Fig. 3.12). Random double deletion strains were chosen from the SGA screen that exhibited deficiency in growth when compared with the single deletions. Spot test on YPD showed no growth of these strains consistent with the SGA results (Fig. 3.12, left panels). Re-introduction of the candidate gene through a vector resulted in growth in all six strains (Fig. 3.12, middle panels). Note that the transformation of these strains with a vector lacking an ORF resulted in the sick phenotype comparable to that of a double deletion strain without a plasmid (Fig. 3.12, right panels).

Figure 3.12. Double deletion gene re-introduction spot test. Confirmation of acetic acid sensitivity as a result of a double knock-out of one of the six candidates and a genetically interacting partner. Double deletion strains were selected producing no growth (left panels). Upon re-introduction of the vector overexpressing the candidate gene, strain exhibits growth at several concentrations (middle panels). Upon introduction of the empty vector (no ORF) into the strain, phenotype is comparable to a plasmid-deficient double deletion strain (right panels). Therefore, we can be sure that genetic interaction data obtained from SGA, SDL and PSA experiments relied on the fact that deletion of two genetically interacting genes may cause a detrimental effect to the strain; sick phenotype was a result of double deletion in the strain and not extraneous effects in the system.

CHAPTER 4. DISCUSSION

Translation initiation is an important rate-limiting step in protein expression. As such, it is subject to regulation in accordance with the cell's functional requirements. IRES-mediated translation is one of the alternative modes that are found to be aiding in protein expression during conditions of cellular stress. The precise mechanisms and elements involved are not well-characterized.

This study focuses on identification of potential components relevant to IRES-mediated translation initiation of heat-shock protein isoforms Hsp82p and Hsc82p. Certain ITAFs have been identified in the past that "moonlight" between IRES-mediated translation and other functions in the cell. It is hypothesized that many ITAFs remain undiscovered that can be identified through establishment of genetic interactions and functional screens. Here we performed a large-scale screen identifying potential ITAF candidates. We then performed SGA, SDL and PSA analysis on the array of translation-related gene deletions to establish the candidates' genetic interaction networks in an attempt to elucidate their functional role in the IRES-mediated translation initiation.

4.1 Identification of the candidate ITAF genes for further analysis

4.1.1 Identification of candidate ITAFs through IRES-mediated β -galactosidase reporter translation

A large-scale screen encompassing an entire yeast non-essential gene library was performed to analyze the effect of gene deletions on the efficiency of IRES-mediated translation initiation, thus identifying potential candidates that may function as ITAFs. For this, a vector containing a *LacZ* reporter with an IRES from *HSP82* or *HSC82* in its 5'-UTR were transformed into a *S. cerevisiae* non-essential gene deletion collection. It was expected that if gene deletion in a

particular strain exhibited a positive regulation effect on the process of IRES-mediated translation, β -galactosidase would not be expressed from the IRES-controlled vector, as one of the activating factors would be absent from the cell system. As a control against the canonical translation initiation, hairpins upstream of the IRES region would abolish normal form of translation completely.^{6,68} Thus, the enzyme would not be produced and would not be able to cleave the X-gal substrate into a blue product. The colony in question would appear white as compared to a negative control (*LacZ* gene under control of a *CLN3* 5'-UTR not containing an IRES). Despite the fact that 48 and 26 such strains were discovered for *HSC82* and *HSP82*, respectively, the overlap consisted of only two genes: *ITT1Δ* and *DOM34Δ*. Same experiments of the prior research on the IRES element of the nitrogen starvation - implicated *URE2* gene yielded numerous candidates (A. Golshani, unpublished data). However, when compared with this study, the overlap in candidates only included the *DOM34Δ* and *ITT1Δ*. This circumstance suggests these two genes to be universal elements governing activity of at least several IRESs, which is not unusual, as certain ITAFs govern translation of multiple IRES-containing genes.^{10,26,69}

The remaining candidate genes were unique to either *HSP82* or *HSC82*, suggesting their function as specific to a particular isoform's IRES. The expression patterns of Hsp82p and Hsc82p were found to be different despite their close homology.^{37,38} Hsp82p variant of Hsp90p is at lower levels in healthy cells when compared to Hsc82p. During stress response, translation of Hsc82p is upregulated. It was also found that the two isoforms have antagonistic functions, Hsc82p protecting cells from stress, while Hsp82p promoting apoptotic processes.³⁸ As well, many of the known IRES elements in eukaryotic cells are regulated by ITAFs unique to them.^{2,10,11,27,28,39} Therefore, it is not surprising that this study identified elements specific to a particular protein variant, tailoring its expression to particular cellular conditions.

It should be noted that when scoring IRES-influencing deletion strains, only very obvious, pure white colonies were analyzed (Fig.3.2). Gradation of color was not scrutinized; thus, colonies of lighter shades of blue were not considered for further study. As well, while performing the lift assay, all plates were incubated at 30°C overnight for the development of color. It was not noted how fast color change took place. Due to these facts, some subtle effects of the enzyme activity, such as partial activation of IRES, or efficiency of translation, were omitted. This represents a limitation of our screen to identify additional candidates that can influence IRES-mediated translation.

Further analysis through the quantitative liquid β -galactosidase assay allowed us to take into account the time it took the cell culture to change colour and the precise amount of substrate cleaved. Only strains identified in the X-gal lift assay were tested for IRES activation through liquid assay. Thus, the precise activity of the enzyme was measured. Majority of the candidates exhibited low β -galactosidase activity when compared to the wildtype, confirming lift assay results and affirming candidate deletions' effect on IRES activity.

4.1.2 Effect of candidate ITAF deletions on cell survival during stress

IRES-mediated translation is proposed to be an alternative mechanism activated during times of cellular stress, including heat shock and chemical toxicity. Therefore, if a particular protein is a positive regulator of IRES, its absence would render the strain sensitive to a particular stressor and result in a lack of growth in hostile conditions. We grew the candidate deletion strains on 3-AT, an inhibitor of histidine biosynthesis and acetic acid. Both substances were meant to simulate starvation conditions. Most of the strains that were sensitive to 3-AT were also sensitive to the acetic acid. The only exceptions are *DOM34 Δ* and *RPS1A Δ* . It was also noted that candidates sensitive in the 30mM acetic acid concentration were also inhibited by 100mM

concentration, but no reverse pattern was observed. This result indicates that strains having sensitivity for 100mM concentration only had higher resistance to perturbations in their environment.

4.1.3 Selection of ATP22Δ, DOM34Δ, MRPS16Δ, RPL36AΔ, VPS70Δ and YBR064WΔ for further analysis

By comparing deletion candidates based on their ability to mediate IRES-based translation and their respective gene ontology, six strains were selected for the follow-up studies. A variety of functions was looked at in the selection process, covering logical links to general translation, IRES-mediated translation, and stress response, such as response to starvation, DNA damage, etc., vacuolar function, and ribosomal activity. Full summary of stress sensitivity and functions associated with each of the studied candidates can be found in Table 3.2. *ATP22Δ* showed no sensitivity to 3-AT but reduced growth on acetic acid, it was considered of interest due to its connection to both mitochondrial translation and DNA replication stress. *DOM34Δ* was selected because it showed decreased β -galactosidase activity in both HSP82 and HSC82 screens, its sensitivity to 3-AT and clear association with several stress response pathways. Mrps16p is a protein that is mainly localized to the mitochondria, it is of particular interest due to its ability to re-localize, same as Atp22p. *RPL36A* encodes a constituent of a ribosomal subunit and is highly sensitive to acetic acid (to both 30min and 200min treatment). *VPS70Δ* showed sensitivity to both 3-AT and acetic acid, is a vacuolar gene linked to many stress pathways. A deletion of a gene with an unknown function (*YBR064WΔ*) exhibited sensitivity to acetic acid and low β -galactosidase activity; was specifically selected in aspiration of identifying its functional significance.

4.2 Genetic interaction networks of the candidate ITAFs and their establishment through SGA, SDL and PSA

4.2.1 Establishment of genetic interactions

Genetic interactions were identified through analysis of deleterious effects on colony growth of the double deletions (SGA analysis) and combination of a deletion of one gene with an overexpression of a candidate ITAF gene (SDL analysis). Deletion of a single non-essential gene may not have an obvious phenotypic manifestation in an organism, as its functional role may be "rescued" by a gene product of another, overlapping, pathway. However, once this gene is also eliminated, the function cannot be carried out and the phenotype manifests itself. Occasionally, when deletion of one gene has no phenotype on cell growth, overexpression of another may create a stoichiometric imbalance of functional units and result in a deleterious phenotype. In either case, communication between the two genes can be classified as genetic interaction. The proteins are not necessarily interacting physically in these scenarios. Rather, a cross-talk between two pathways is indicated. Through large-scale experiments incorporating an entire collection of non-essential gene deletions, it is possible to screen 80% of the yeast genome for the genetically interacting partners of a gene of interest, thus identifying novel elements omitted in prior studies. Further modification of the assay by measurement of the strain growth would complement the study by quantitatively confirming the list of potential interacting partners.

This research is focused on establishing the six candidates' genetic interactions and analyzing their prospective function as ITAFs. The amount of data generated by such large-scale study is vast, this is why we narrowed down the array from the entire non-essential genome collection (~4800 genes) to only the genes involved in the protein biosynthesis (~380 genes). SGA screen was performed combining this deletion array with the candidate deletions and growing the

resultant strains in optimal conditions (30°C, YPD), and with stress induction (conditional SGA). As well, vectors over-expressing the six candidates were transformed into the translation array and grown in optimal and stressful conditions for SDL and PSA analyses.

4.2.2 Conditional studies and the importance of mTOR pathway

It is generally accepted that IRESs are often utilized under stress conditions. In this research, conditions were used to cause a stress to a cell system, activating stress-response pathways, including IRES-mediated translation mechanism. Importantly, treatments were selected to induce the cell "fight" for survival in inhospitable conditions, while not so much as to promote apoptosis. Thus, sub-inhibitory concentrations of reagents were used, and for the heat shock treatment, temperature was raised to only 37°C. Weak acids such as acetic acid has long been used in similar experiments as an inhibitor of fungal growth.^{38,70} As with other sources of stress in *S. cerevisiae*, when subjected to sub-inhibitory concentrations of acetic acid, translation becomes attenuated, thus only proteins essential for survival in hostile environment are translated. Other chemicals used in this study include cycloheximide (inhibitor of tRNA translocation) and paromomycin (binds 16S ribosomal RNA). Of particular interest is the rapamycin treatment, as this chemical is a specific inhibitor of translation initiation. Its mode of function involves repression of a protein complex TORC1 in the mTOR pathway responsible for maintaining homeostasis during stress.^{71,72} Upon binding rapamycin molecule, TORC1 is inhibited from phosphorylating protein 4E-BP1, which is bound, as the name suggests, to eIF4E, inhibiting the latter from carrying out its essential role in canonical translation initiation.⁷¹ In this manner, rapamycin directly contributes to repression of canonical translation initiation and may contribute to activation of IRES-mediated translation in treated strains. As will be discussed, mTOR pathway was identified in multiple candidates' networks.

Once genetically interacting partners for each potential ITAF were identified through SGA, SDL and PSA, their functions were analyzed. Associations with stress response and translation pathways were favorable as links to IRES-mediated translation initiation.

4.2.3 GO analysis of mitochondrial ATP22Δ and MRPS16Δ genetic interactions

Atp22p is a factor involved in mitochondrial protein biosynthesis.^{73,74} It is translated in the cytoplasm and is imported into the mitochondria to fulfill its function. Atp22p possesses a hydrophobic region which anchors it to the inner mitochondrial membrane.⁷³

Analysis of *ATP22Δ* SGA implicated the gene in stress response pathways through strong genetic interaction with *SGS1* (Table 3.5), which, among other stress-response related functions is a repressor of the rapamycin responsive genes (Table 3.3).⁶³ SDL and PSA screens produced similar results, though stress response pathways were less enriched (association with *YOL115W*, protein of diverse functions including RNA degradation and DNA base excision repair (Table 3.10)).⁷⁵ Association of a mitochondrial gene with stress response is logical, as mitochondrion is highly involved in stress response of many kinds (nutrient, oxidative, chemical-induced), and certain mitochondrial ribosomal proteins are implicated in pathways outside mitochondrial translation.^{71,76} For instance, mitochondrial protein DAP3 (human homologue of yeast Rsm22p) is both part of a small ribosomal subunit and has a role in apoptosis activation.⁷⁶ It induces mitochondrial fragmentation, resulting in membrane permeabilization and release of cytochrome c and caspases which proceed to activate apoptotic mechanisms.^{77,78} However, Dap3p still acts from within the mitochondria and becomes activated when the cellular system cannot be saved and apoptosis ensues.

On the other hand, a mitochondrial factor that is actually working to diminish cellular stress and help regain homeostasis is Ymr31p. Studies indicate that the mammalian homologue

mMrps36p, apart from its role as a ribosomal constituent is involved in cell cycle arrest by altering p53 expression.⁷⁹ Note that unlike Atp22p, mMrps36p has no hydrophobic domain and has been localized to both cytoplasm and the mitochondria in this research.⁷⁹ While localized inside the mitochondria and isolated from the cytoplasmic translation machinery, Atp22p is unlikely to exhibit a function of an ITAF. Since localization of Atp22 was determined during optimal cellular conditions,⁸⁰ it is possible that during stress it remains in the cytosol, stabilized by its respective chaperone, instead of becoming imported by alteration of mitochondrial import mechanisms.

Mitochondrial import of cytoplasmic proteins relies on the translocase of outer membrane (TOM) complex, which is known to be phosphorylated on several serine and threonine residues by cellular kinases in accordance with the cell's metabolic needs, this affects its affinity for the proteins targeted for import.⁸¹ Possibly, during certain types of stress, TOM complex becomes phosphorylated, abrogating its interaction with Atp22p and its chaperones, which then become redirected to regulate the IRES-dependent translation initiation.

An association was established in the SGA, SDL and PSA screens between *ATP22* and the cytosolic as opposed to mitochondrial translational elements (Table 3.3, 3.4). The implication is more unusual, since it is unclear how mitochondria-anchored Atp22p can exhibit any functional overlap with the cytosolic translation pathway.

Another protein involved in mitochondrial translation, Mrps16p, is also encoded by the cellular genome. It is translated in the cytosol and then imported into the mitochondrion.⁸¹ It is part of a small ribosomal subunit that is highly conserved between yeast, humans and *E. coli*.⁸² In humans, mutations of MRPS16 result in severe congenital disorders, though mainly directly due to malfunction in the mitochondrial respiratory chain.⁷⁶ Unlike *ATP22*, *MRPS16* was not

studied extensively. Apart from the protein's direct role in mitochondrial translation, no novel functions have been identified. Although localized to the mitochondrion, it is a soluble protein, potentially able to exist in a fully-functional form in the cytoplasm, akin to previously described mMrps36p. It is possible that during stressful conditions translated Mrps16p is no longer transferred into the mitochondria via TOM complex, but becomes redirected to the translation machinery to aid in restructuring IRES sequence or take place of inhibited eIFs.

MRPS16Δ was found to have negative genetic interactions with eIF5A, component of both cytosolic initiation and elongation steps, as well as translation inhibitor *EAP1* (Tables 3.3, 3.4, 3.7). It also has associations with proteins involved in nucleosomal rearrangements (Fig.3.8), which show genetic and physical associations with vacuolar genes further linking *MRPS16* to stress response pathways. Therefore, this study identified Mrps16p links to both stress response pathways consistent with mitochondrial function and cytosolic translation, establishing it a viable candidate to IRES function.

4.2.4 GO analysis of DOM34Δ genetic interactions, implicating stress response and protein biosynthesis

Dom34 is a well-characterized protein involved in regulation of translation mechanisms. Dom34p functions in a complex with Hbs1p; the pair is comparable in structure and function to eukaryotic translation release factors eRF1 and eRF3, respectively, acting as a complex to execute translation termination.^{16,83,84} Dom34-Hbs1 complex binds the 60S subunit and acts in concert with other factors to promote subunit dissociation in an ATP-dependent manner.⁸⁴ This mechanism exists as a quality control step, ensuring proper assembly of the ribosomes on mRNA prior to the elongation commencement.^{84,85}

GO analysis of *DOM34Δ* genetic interactions exhibited high enrichment of the mTOR pathway. Our prior knowledge of the Dom34p-Hbs1p function helps to explain this connection, as this

complex was found to be triggered by the stalled machinery during translation elongation stress caused by circumstances such as nicks or stable secondary structures in mRNA, misfolded translation or canonical release factors, etc.^{83,86} Studies also implicated Dom34-Hbs1 complex in release of ribosomal subunits during stress-induced translation inhibition.^{83,84,86} Since stress-induced inhibition of TORC1 is directly linked to translation inhibition and subsequent ribosomal subunit dissociation, our findings are consistent with the known functioning of the protein.

Another gene contributing to *DOM34* interaction network was *STM1*. As in the mTOR example above, this association is in accordance with our current knowledge about Dom34p. Stm1p plays a major role in translation inhibition during stress. The dissociated ribosomes form a pool of 80S ribosomal complexes that are held together by Stm1p. The protein both prevents 80S-mRNA interactions and protects ribosomes from degradation. Upon relief of cellular stress, Dom34p-Hbs1p are called to break up the 80S complex releasing the ribosomal subunits such that protein synthesis can quickly recommence.⁸⁴

Several novel associations with *DOM34* were discovered through this study. For instance, protein Gsy1p was identified. It is known to interact with kinase Pho85p involved in countering various cellular stresses.⁵⁵ Another factor, Bsc1p, has no confirmed function, but a similarity to Flo11p, a protein involved in biofilm formation. Considering biofilm formation is often exhibited by cell cultures as a collective response to hostile environment, our data concurs with the prior studies.^{87,88}

In conclusion, Dom34p has been implicated in regulation of protein biosynthesis and stress response by prior studies. Our data concurs with the research, since we identified previously described genetic interactions (*TOR1*, *STM1*, ribosomal genes), and since novel associations are

also linking the candidate to stress response. Potentially, during stress Hbs1p-Dom34p complex is influenced by phosphorylation to dissociate, leaving the latter protein with an altered conformation. Dom34p can then aid in ribosomal recruitment to the IRES site or act like a molecular bridge between other proteins involved in translation initiation.

None of these associations would contradict potential characterization of Dom34p as an ITAF. However, they could be easily explained by its currently known role of a non-canonical release factor. Despite Dom34p's apparent indispensability in IRES-mediated translation initiation of both HSP82 and HSC82 5'-UTRs, further studies are required to confirm this protein's role in non-canonical translation initiation.

4.2.5 GO analysis of cytosolic ribosomal RPL36AΔ genetic interactions

RPL36A codes for one of the proteins of the 60S ribosomal subunit. As with many other ribosomal proteins, mutations have been linked to cancer. A recent study proposed it to function as a tumor suppressor in hepatocellular carcinoma, though the mechanism of action is yet to be determined.⁸⁹ The SGA screens showed enrichment of various translation-related pathways, such as ribonucleoprotein granule ($p=2.47E-02$, acetic acid) and translation elongation ($p=1.10E-02$, SGA). SDL analysis also exhibited this candidate's interaction with *SBP1*, a protein involved in translation repression (Table 3.10). Sbp1p is known to facilitate decapping and further degradation of the mRNA through canonical translation factor eIF4G binding.^{67,90} These interactions directly link Rpl36ap to the translation process and the mechanisms of its regulation, which corresponds perfectly with the protein's primary role as a ribosomal constituent.

Another direct link to the translation pathway, *ZUO1*, demonstrated genetic interactions with the *RPL36AΔ*. Zuo1p is a co-chaperone for nascent polypeptide folding, suggested to interact

with the ribosomes during the translation process via rRNA binding.^{91,92} At the same time, a variety of other functions were suggested for this protein, including a role in ribosomal biogenesis, and conveying resistance to osmolarity, low temperatures and paromomycin.^{91,92} The latter detail is in accordance with our experiment, since *ZUO1Δ* was identified in screens involving 5 different types of stress, providing a stronger connection between *RPL36AΔ* interactions and both translation and stress response.

Translation-related *TAE2* was identified via rapamycin screen. A part of the Ribosomal Quality Control (RQC) it identifies and targets aberrantly-translated nascent proteins for degradation.^{65,93} This complex is also responsible for signaling the presence of translational stress.⁶⁵ From all of the above examples (*SBP1*, *ZUO1*, *TAE2*), a consistent connection is evident between ribosomal *RPL36A* and the translation and stress response pathways.

Further analysis of *RPL36A* interactions identified more strong associations with genes directly involved in response to particular kinds of stress. For instance, *PDR1* was found, a master regulator controlling a family of genes responsible for efflux of drugs from the cell.⁹⁴ Another unusual example is *SWI3*, product of which is part of a SWI/SNF complex of chromatin remodeling factors regulating gene expression in accordance with the perturbations in cellular conditions.⁹⁵ In hypoxia Swi3p, along with several other proteins, is localized in cytosol, leaving remainder of the complex to activate hypoxia-specific genes. Meanwhile, Swi3p binds Cln3p, thus inhibiting cell cycle progression from G1 to S phase.⁹⁶ Upon re-establishment of normoxic conditions, cytosolic proteins re-localize from the cytosol, combine with their nuclear-specific associates and re-establish expression patterns appropriate for the optimal cellular conditions.^{95,97} This particular interaction is more directed at response to cellular stress and cell cycle control and is more unusual for a ribosomal protein. Considering IRES-mediated translation is

upregulated during stress, however, this interaction network may indicate a greater probability of Rpl36ap being an ITAF.

Another chromatin-associated gene was identified in this screen - *HHT1*, encoding a histone H3. Modifications to histone tails lead to decondensation of the DNA, allowing accessibility for transcription of various gene groups, as well as DNA damage repair.^{98,99} Although size reduction of the colony with double deletion *RPL36AΔ/HHT1Δ* was only 35% (Table 3.8), and the association is unusual for a ribosomal protein, connection to the important regulatory functions such as chromatin remodeling add to relevance of the candidate *RPL36A* as a potential ITAF.

In general, *RPL36A* screen demonstrated a very diverse interaction network with rather baffling associations. For instance, strong genetic interactions were established with the *YDR282CΔ* (74% colony size reduction). The latter is a gene of unknown function that was selected earlier in the study as one of the potential IRES-regulating candidates (Table 3.2). The protein product of this gene has been localized in the mitochondria; it only recently attracted attention when a mutation in the human homologue was implicated in severe birth defects due to imbalanced enzymatic function within the mitochondria.¹⁰⁰ We have identified a connection between mitochondrial candidates *MRPS16* and *ATP22* and ribosomal translation machinery earlier. Such a strong interaction between the mitochondrial and the cytoplasmic ribosomal proteins keeps appearing throughout this work, though it is unconventional.

Also, GO analysis showed an enrichment of the factors involved in the degradation of cellular factors. For instance, gene *PRC1* was identified, encoding Carboxypeptidase Y (CPY), a serine hydrolase. This protein is localized in the vacuole, being responsible for protein degradation as a result of autophagy, endocytosis, as well as the proteins mislocalized from the secretory

system.^{101,102} Another enriched protein involved in the recycling of cellular elements was Lsm1p. As part of the cytosolic Lsm1p-Lsm7p complex, it is responsible for decapping and degradation of the mRNAs.^{103,104} It is also known to shuttle to the nucleus where it acts as a transcription factor, activating genes involved in mRNA degradation, and contributing to hydroxyurea stress response^{103,105} It can be seen that these associations (*YDR282C*, *PRC1*, *LSM1*), although random, are all converging at the stress response pathways, linking the candidate *RPL36A* to regulation of cellular stress.

Overall, gene *RPL36A* demonstrates affiliations with a wide variety cellular pathways, including the ones regulating response to stress. Even though such interactions are characteristic of many ribosomal proteins, it could potentially act as an ITAF modifier as well as being part of the canonical translation initiation. As part of a ribosomal complex, Rpl36ap may be instrumental in recruitment of the 60S subunit to the initiation complex. Alternatively, it may aid in keeping 60S in a free-floating form while the other subunits are held in 80S dimers by Stm1p. These theories can be explored to fully characterize this protein's role in translation.

4.2.6 GO analysis of vacuolar *VPS70Δ* genetic interactions

Gene *VPS70* has not been well-characterized by prior research. In this screen it demonstrated strong interactions with cytosolic ribosomal components, resultant in >80% growth reduction on acetic acid and rapamycin (*RPL9A* & *RPS9B*, Table 3.9). If we consider an intricate functional overlap in the processes that take place during starvation conditions, this association can be logically explained.

In general, vacuole is an important organelle for protein recycling in yeast, analogous to mammalian lysosomal compartments.^{106,107} During conditions of cellular stress, in particular conditions mimicking starvation, yeast cells are able to undergo the process of autophagy - they

recycle some proteins and inorganic compounds in the cytosol into simpler components crucial for survival.¹⁰¹ Translation initiation of most proteins is stalled in such conditions and all components become either temporarily inactivated or recycled by autophagy.^{10,28,108} Vacuolar proteins play a major role in this process. Meanwhile, certain initiation factors and 40S ribosomal subunits become accumulated in the stress granules (SGs), implicating these aggregations in translation of proteins essential for survival during stress through unconventional translation modes, for example IRES-mediated.⁷² Thus, research shows that the processes of autophagy and stress-specific translation overlap, justifying our findings and suggesting a realistic role for Vps70p as an ITAF in IRES-mediated translation initiation.

Interactions were evident between *VPS70* and some regulatory proteins, such as NUP84, part of a nucleopore complex. Possible connection between NPC and Vps70p, however, is not clear. Research shows that both import of proteins into the nucleus and export of proteins and RNA out into the cytoplasm are carefully regulated.¹⁰⁹ It has been found that during starvation the TORC1/2 complex promotes hypophosphorylation of transcriptional factors, directing their localization into the nucleus to activate the genes responsible for assimilation of poor nitrogen sources. In order to gain entry through the nuclear pore, these TFs must bind the specialized transporters associated with the NPC, including NUP84.¹¹⁰ It is possible for Vps70p to function as an ITAF as described upstream of these events. It should be noted that despite being associated with starvation conditions, *NUP84* was identified in *VPS70Δ* screen through regular SGA and heat shock treatment, although it was present as an interacting partner in conditional screens of *ATP22Δ* (35.84% reduction; SGA & acetic acid) and *RPL36AΔ* (26.32% reduction; paromomycin).

As a part of the vacuolar processes, it is not surprising to find Vps70p display genetic interactions with proteins involved in regulation of various stress response pathways. More unusual is the presence of multiple translation and nuclear pore-related factors in the interaction network. Connections between these functions and *VPS70* are not clear, since implication in multiple processes is a common characteristic of ITAFs, this candidate is a viable candidate for regulation of IRES-mediated translation. Being linked to the formation of stress granules, perhaps Vps70p is involved in recruitment of the 40S subunits to the IRES sequence. Additionally, it may aid in restructuring of the IRES to aid in ribosomal binding. Further studies are necessary to clarify this protein's function.

4.2.7 GO analysis of uncharacterized *YBR064W*Δ genetic interactions

YBR064W is a gene of unidentified function overlapping another ORF, *YBR063C*, which encodes a putative non-essential protein of unknown function.⁶⁰ *YBR064W* is considered to be a dubious ORF not encoding a functional protein product. Despite the fact, null mutant screen produced some interesting interactions with ribosomal elements and some regulatory networks. The former consist of ribosomal proteins of large and small subunits, *RPL23A* and *RPS17A*, as well as *NOP16*, a nucleolar protein involved in 60S subunit biogenesis.¹¹¹ All three exhibited strong genetic interactions with *YBR064W* (>70% size reduction, Table 3.9).

Some interesting examples of quality - control genes were identified to have genetic interactions with *YBR064W*. *PAP2*, for instance, encodes a protein involved in modulation of the nascent non-coding RNAs.⁷⁵ Pap2p is a catalytic subunit, together with RNA-binding proteins constitute a TRAMP complex responsible for quality control of the newly-synthesized tRNAs and rRNA precursors.^{75,112,113} The complex recognizes aberrantly folded RNA molecules and targets them for degradation by polyadenylation.^{75,112} In more recent studies, TRAMP complex has been

linked to DNA repair via base-excision repair pathway.^{113,114} The latter finding expands the role of TRAMP in the nucleus, since perturbed chromatin structure tends to give rise to transcription of cryptic non-coding RNA, which is also recycled by the TRAMP complex.¹¹³ Considering *YBR064W* has no previously identified function, well-researched associations, or localization, the connection between it and *PAP2* cannot be interpreted by current knowledge. However, the implication of TRAMP complex in the DNA damage repair and RNA degradation may indicate the association between the candidate and stress response which is favorable for its putative role as an ITAF.

Strong genetic interactions with the candidate were exhibited by *SAC3* (70.36% reduction; acetic acid) (Table 3.9). Sac3p is a protein of multiple functions localized to the nucleus. Transcription, modification and export of RNA from the nucleus are very closely linked and Sac3p appears to have a role in all of these processes. Sac3p functions as part of a complex called THSC. This assembly has recently been implicated in transcription elongation, though the influence is not entirely elucidated.¹¹⁵ As well, THSC assists in tethering of newly transcribed mRNA in aggregations near the transcription sites as a result of errors in biogenesis of their RNP chaperones.^{116,117} Interestingly, on its own Sac3p is localized both inside the nucleus, as well as to the cytoplasmic component of the nucleopore, since it is also involved in the last stage of export of mature mRNA from the nucleus.¹¹⁷ As in the previous interaction, connection between the *YBR064W* and Sac3p is unclear, though this protein may be implicating the candidate in the regulatory process associated with one or all of its functions.

As well, *YBR064W* was linked to cell cycle control through genetic interactions with *CLN3*, a cyclin regulating the G1-S phase transition. Cln3p is transcribed throughout the cell cycle and is regulated post-transcriptionally. During nutrient starvation the mTOR complex represses

translation of all proteins, including Cln3p.¹¹⁸ Upon stress relief the latter is translated, it induces transcription factors to activate cell cycle progression genes, allowing the cell to exit G1 and enter the S-phase.^{118,119} Once again, the regulatory behavior of the interacting partner during adverse cellular conditions may be linking the candidate to the stress response regulation pathways.

Overall *YBR064W* is one of the more difficult genes to analyze, due to a complete lack of prior research. Despite the fact, it exhibits numerous genetic interaction patterns (Fig.3.10), suggesting it to be an interesting candidate for a multifunctional protein as most ITAFs are.

4.3 Phenotypic suppression analysis links candidate ITAFs with translation and stress response pathways

Apart from SGA and SDL, we can establish genetic interactions of the candidate genes by over-expressing them in a strain gene deletion of which confers sensitivity to one of the five stressors (heat shock, acetic acid, cycloheximide, paromomycin and rapamycin). The same strain may exhibit healthy growth on regular media in optimal conditions. Phenotypic suppression of a sickness by an over-expressed protein in such cell culture indicates genetic interaction between the removed and the over-expressed elements. PSA was performed combining over-expressed candidate genes with the translation deletion array. We used this approach, similarly to SGA and SDL, to look for diverse interactions providing a valid link between the candidate and IRES-mediated translation initiation.

It was found that most of the pathways enriched in SGA were also enriched in this experiment, though less diversity was observed. Table 3.10 shows that most of the GO processes involve translation regulation and ribosomal biogenesis and processing. Although not providing any

unexpected, novel data, this screen supports the associations previously identified in this study between our candidates and the protein expression pathway.

Some genes were found to overlap between the SGA and the PSA screen. For instance, *HSP30* was identified through both *DOM34Δ* acetic acid-conditioned SGA and *DOM34Δ* acetic acid-conditioned PSA screens (Table 3.11). In this situation we see deletion of genes *HSP30* and *DOM34*, separately, producing no visible phenotype in optimal conditions. When subject to acetic acid conditions, *DOM34Δ* is growing, but *HSP30Δ* is sick. A combination of *DOM34Δ/HSP30Δ* in a strain grows at optimal temperatures, but when subjected to acetic acid exhibits a sick phenotype. Overexpression of gene *DOM34* rescues the healthy phenotype exhibited by *HSP30Δ* in presence of acetic acid. Such association appears to show strong genetic interactions between genes *HSP30* and *DOM34*. All this pattern really exhibits is a validation of connections between the functions performed by these genes and our candidates.

Of the 28 genes exhibiting this pattern, 6 were ribosomal. One of these, *RPS9B*, was associated with no less than four candidates (Table 3.11). Presence of so many ribosomal genes adds to the validity of the screen, as it links candidate ITAFs with the translation pathway.

Other genes were identified as part of PSA to link candidates with the stress. *ASC1* is involved in response to starvation and DNA replication stress.¹²⁰ Both *GLC3* and *SIP4* are activated during glucose starvation. Interestingly, some heat shock proteins were identified multiple times, including *HSC82* itself. The latter was identified to interact with *YBR064WA*, which is unusual, since the β -galactosidase test identified the candidate to influence Hsp82p expression, but not Hsc82p. This result may indicate the candidate ITAF regulating expression of Hsp82, while being involved in a parallel pathway with Hsc82p.

Phenotypic suppression analysis allowed us to identify more genetically interacting partners of the candidate deletions through a slightly different approach as compared to the SGA and SDL. We found that PSA screen is consistent with our prior experiments, providing a link between the ITAF candidates and mechanisms of translation and stress response. Of special interest were the genetic interactions identified in the same strains and conditions of SGA and PSA, as they were directed to, and succeeded in, validating our previous findings.

4.4 Re-introduction of candidates in their respective deletion strains rescues sensitivity to chemicals

In this study we looked at the interactions between the two genes based on the phenotype exhibited by the combinations of their deletions in the strain. Controls have to be used to make sure the phenotype exhibited by the strain is specifically due to the absence of the studied gene and not due to certain unanticipated secondary mutations. Additionally, we have to ascertain that sick phenotype of a double deletion strain is the result of a genetic interaction between studied genes, and not due to extraneous effects. For each single candidate deletion strain, a vector was introduced overexpressing its corresponding gene and a spot test was performed on YPD media and in presence of acetic acid (Fig.3.11) and each of the studied antibiotics (data not shown). As well, an empty vector was transformed into all deletion strains for a comparison (Fig.3.11). Initially, all strains exhibited normal growth on YPD, while showing sensitivity to the chemicals. Upon re-introduction of the gene, the normal growth phenotype in presence of chemicals was rescued. Strain transformed with an empty vector exhibited no growth, supporting the idea that sick phenotype was a result of a gene deletion.

Similar experiments were performed on double deletion strains (Fig. 3.12). A vector encoding an over-expressed gene was transformed into a strain with a corresponding gene knocked out

along with a deletion of a genetically interacting partner. A spot test was performed on YPD, and an empty vector was used as a control alongside the over-expressing plasmid. As in the previous scenario, strains exhibiting a sick phenotype were rescued by over-expressing the candidate ITAF genes. Strains transformed with an empty vector showed lack of growth comparable to the vector-free strain.

These experiments validated the results of SGA, SDL and PSA analyses by showing that the phenotypes exhibited by the strains studied were a result of protein dosage, as encoding genes were synthetically over-expressed or removed from the cells and not influenced by any extraneous effects.

CHAPTER 5. CONCLUSIONS

As a rate-limiting step in the process of protein biosynthesis, translation initiation is subject to the highest degree of regulation. Errors in mechanisms governing translation initiation are implicated in development of diseases and cancerous formations. Being an introductory step in the process, multiple variations exist on the canonical mechanism that can be activated in accordance with cellular requirements.

A focus of this study is to further investigate the genetics of IRES-mediated translation initiation, a mechanism that has been well-studied in recent years, but is still mysterious for its complexity of regulation as well as the multitude and variety of proteins involved. Of these, certain canonical factors are known to be involved in some cases of IRES-mediated translation. Of a particular interest, however, are ITAF elements, proteins possessing multiple functions within the cell that are redirected to assist with translation initiation by various cellular stimuli, such as sensors of cellular stress.

Due to this connection between activation of ITAF elements and stress response, induction of stress on the analyzed system was an important aspect of this study. During environmental stress, be it starvation, anoxia or chemical toxicity, cellular metabolism undergoes drastic changes affecting many pathways to reduce energy waste and direct it towards initiating and maintaining the processes allowing the cell to survive inhospitable conditions. Protein biosynthesis pathways are affected in these circumstances. It has been shown that majority of protein synthesis initiated by canonical means becomes abrogated, only those elements essential for cellular recovery are synthesized through alternative methods, including IRES-mediated translation, which are dependent on ITAFs.¹⁰

We hypothesized that many of these elements remain undiscovered and tried to identify them by establishing each candidate's genetic interaction networks and analyzing these to elucidate the potential ITAF's plausible function in IRES-mediated translation mechanism. We used the *S. cerevisiae* non-essential gene knock-out library to screen for deletions affecting expression of a reporter construct under IRES control. We identified over 40 candidates potentially involved in IRES-mediated translation of proteins Hsp82p and Hsc82p. Six candidates were selected for follow-up studies: *ATP22Δ* (*YDR350CΔ*), *DOM34Δ* (*YNL001WΔ*), *MRPS16Δ* (*YPL013CΔ*), *RPL36AΔ* (*YMR194WΔ*), *VPS70Δ* (*YJR126CΔ*), and *YBR064WΔ*. Their genetic interactions were determined and analyzed. It appears that all of these genes have associations with the translation pathway, and most of them are linked with at least one kind of stress response, supporting their role as potential ITAFs.

Further research in the field should be directed at elucidating these six candidates' precise functions in IRES-mediated initiation mechanism. The remaining *HSP90*-specific candidates should also be screened for genetic interactions and analyzed analogously to this study.

Since many ITAFs were shown to be specific to a particular protein, large-scale studies should be performed for identification of other such elements and analysis of their function.

APPENDIX I

Table I. Sequences of primers used for gene knockout. Forward and reverse sequences are shown used to achieve homologous recombination with the chromosome, as well as confirmation sequences ≤ 1 kb upstream of the gene and within the Nourseothricin resistance gene utilized in confirmation PCR. Ordered from Integrated DNA Technologies.

Gene Name	Primer Description	Sequence
<i>ATP22</i>	forward	5'- CGT ACT GGC AAG AAT GGG CTT TCA AAT CTT CCA AGC GTT CAC TTT AAT GTT CTT ATT GGC CAC ATA CGA TTT AGG TGA CAC -3'
	reverse	5'- TTA TGG GTA AAT TAC TAT CTA TGA ATG AAT ATT TAC TAT TTA CTA GTG CTC ATC TGG ATA AAT ACG ACT CAC TAT AGG GAG -3'
	confirmation	5'- CTA GGT TGG AGC TAG GGC GTC TTG -3'
<i>DOM34</i>	forward	5'- TTA AGC ATT CGT TGC TGC ATC GTT GTC ATT TTG TTC AAT TAT CGC ATT CCT ATC ATA GCA CAC ATA CGA TTT AGG TGA CAC -3'
	reverse	5'- CTG CAC GTG ACT AAC TAG AAC CAT TTC ACA ACA ACT CAA TTA ATT GCG GCT TCA CAT CTA AAT ACG ACT CAC TAT AGG GAG -3'
	confirmation	5'- GTC GAC AGA GTT CTC TTC GGC GCC ATT AGC -3'
<i>MRPS16</i>	forward	5'- AAG TAA CGG AGG AGT TCT GAC TGG TAT TCC GTT AAG AAA TTG TAA TAG AGT TTA TCA AGT CAC ATA CGA TTT AGG TGA CAC -3'
	reverse	5'- AAA GGT AAA TAG TGC CGA ACA GTC AGG AGC ACA CAA TAC ATG ACC TAT AAT GAC ACT ATC AAT ACG ACT CAC TAT AGG GAG -3'
	confirmation	5'- CTG TGC GCA CTC TAC GCT GAT CGC TTG C -3'
<i>PCS60</i>	forward	5'- TTG AAA TTT ACT CCC ACT GTG CTG CAA GAG GTT AGA CGC TTC ACG CAC ATT TTT GCT ACA CAC ATA CGA TTT AGG TGA CAC -3'
	reverse	5'- GTA TAT AAA CGG GTG CCA GAT AGA AGC TTT CAG AGA GCA TAA AAT TGT ACA GGA TAC TGC AAT ACG ACT CAC TAT AGG GAG -3'
	confirmation	5'- CCA AAC AGC ATG GGG CAC CGT TTC -3'
<i>RPL36A</i>	forward	5'- CAG TAT AGT ACT GAT TTA CAA GTA TCA AAG AAA CAA GAA AGA CAG CAA ACA CAA TAA GAA CAC ATA CGA TTT AGG TGA CAC -3'
	reverse	5'- CTA AGT TAA AAA GAA AAT ATG ATA TCT ATA TAT ATA TTA CAA AAA TCA GTT TTT AGA AAC AAT ACG ACT CAC TAT AGG GAG -3'
	confirmation	5'- GAG AAT CCC CCA CAT AAG GAT GCA -3'
<i>VPS70</i>	forward	5'- GCA AGG GGC CGT ACT TAT TAA AAC AAT TTC AAC ATG CAC AAT TAA GAA TGT AAG AGA TAT CAC ATA CGA TTT AGG TGA CAC -3'
	reverse	5'- AAG TCA CTC TAC GTA TGC AAA TAA AAT AAA TGT ATA TAC TGA GGT GAG GTA CTT AAA TGC AAT ACG ACT CAC TAT AGG GAG -3'
	confirmation	5'- GAC AAG GCA AGG GGC CGT ACT TAT -3'
<i>YBR064W</i>	forward	5'- TTT CTC TCT TCG GTA ACG TCA CTT CGA TTA TTG AGG CAG TCT TCA TTA AAG TTT ATT ACA CAC ATA CGA TTT AGG TGA CAC -3'
	reverse	5'- AGG TAC ATG ATA TAT TGA CTG ATT TCT GAT AGC TCT TTT TTT TCC AAT ATC GAT AAA AAA AAT ACG ACT CAC TAT AGG GAG -3'
	confirmation	5'- CGG GTA GGA GAG TTG GTC ACC AAA -3'
<i>NatR</i>	confirmation	5'- TCC AGT GCC TCG ATG GCC TCG GCG -3'

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