Functional Genomics in Yeast

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Abstract

Living cells are complex biological systems with large networks of interactions between different macromolecules. A focus of systems biology is to study the dynamics of the living cells and their interactions through functional genomic approaches. High-throughput techniques and whole-genome screening experiments allow us to collect “big data” for various biochemical networks within the cell. The baker’s yeast, *Saccharomyces cerevisiae,* is a single-cell eukaryotic model organism used for functional genomics approaches, the genome of which has considerable homology with the human genome. In this thesis, we used functional genomic approaches to study novel function(s) for genes and pathways affecting translation. The process of translation is an essential pathway that leads to the production of functional proteins for living cells. Dysregulation of this process and its associated pathways have been linked to many diseases, emphasizing the importance and need to investigate details of genes that influence this pathway. Much has been learned so far but there are still unknown regulations that require more examination.

One of the main objectives of this thesis is to discover and study novel function of genes that affect the translation of structured mRNAs in yeast. We designed different constructs to evaluate the effect of nearly 5000 non-essential genes on the translation of reporter genes. Through whole-genome screening experiments and follow up assays we proposed the heretofore unknown involvement of five genes in the translation pathway. In the current thesis, *YTA6*, *YPR096C*, *NAM7*, *PUS2,* and *RPL27B* are proposed to be

important for the translation of mRNAs with structured regions within their 5'-UTRs in yeast. In another study, we used translation in mitochondria as a strategy to investigate the ability of a specific 3'-UTR sequence to direct a reporter mRNA into yeast mitochondria. Lastly, using additional screenings we propose the involvement of dozens of more genes that seem to be important for the translation of structured mRNAs.

Together, our findings contribute to a better understanding of the translation of structured mRNAs. They also indicate that our overall understanding of the regulations of translation may require additional studies in years to come.

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Statement of contribution

Chapter 2: Sensitivity of yeast to lithium chloride connects the activity of *YTA6* and *YPR096C* to translation of structured mRNAs.

"Sensitivity of yeast to Lithium Chloride connects the activity of *YTA6* and *YPR096C* to translation of structured mRNAs", is the result of experiments primarily designed and carried out by myself. A group of undergraduate and graduate students assisted me and worked under my supervision. Narges Zare, Sarah Takallou, Tom Kazmirchuk, Nathalie Puchacz, and Sasi Jagadeeson helped me with large scale and small scale *β-galactosidase* experiments and screenings. Houman Moteshareie, Mohsen Hooshyar, Katayoun Omidi, and Daniel Burnside helped with SGA and PSA analysis. I wrote the manuscript.

Chapter 3: Lithium chloride toxicity is connected to the regulation of gene expression in yeast.

"Lithium chloride toxicity is connected to the regulation of gene expression in yeast", is the result of experiments primarily designed and carried out by myself. A group of undergraduate and graduate students including Kathryn Hunt, Grace Kirby, and Sasi Jagadeeson helped me with large scale and small scale *β-galactosidase* experiments and screenings. Sarah Takallou and Katayoun Omidi helped me with SGA and PSA experiments. I wrote the first draft of the manuscript.

Chapter 4: A correlation between 3'-UTR of *OXA1* gene and yeast mitochondrial translation.

"A correlation between 3'-UTR of *OXA1* gene and yeast mitochondrial translation", is the result of experiments primarily designed by myself and Noor Sunba. A group of undergraduate and graduate students helped me with *β-galactosidase* experiments including Sarah Takallou, Nathalia Puchacz, Houman Moteshareie, and Sasi Jagadeeson. Nazila Nazemof contributed to running the qRT-PCR experiments. Drug treatment optimization was performed by me with the help of Katayoun Omidi and Daniel Burnside. Mohsen Hooshyar contributed to analyzing the results and writing the manuscript. The manuscript was written by me.

Chapter 5: Translation regulation of highly structured 5'-UTR constructs.

"Translation regulation of highly structured 5'-UTR constructs", is the result of experiments primarily designed and carried out by me. Sarah Takallou and Sasi Jagadeeson helped me with *β-galactosidase* experiments and screenings. I wrote the initial manuscript for this project.

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Abbreviations

ATP	Adenosine Triphosphate
BD	Bipolar Disorder
cDNA	Complementary DNA
NAT	Nourseothricin Sulfate (clonNAT)
eEF	Eukaryotic Elongation Factor
eIF	Eukaryotic Initiation Factor
FFE	Free Flow Electrophoresis
GAL	Galactose
GI	Genetic Interaction
GSK-3	glycogen synthesis kinase-3
G418	Geneticin
IC	Initiation Complex
IRES	Internal Ribosome Entry Site
LB	Lysogeny Broth

LiCl	Lithium Chloride
MAT	Mating type locus
mtDNA	mitochondrial genome
mRNA	messenger RNA
ONPG	O-Nitro-Phenyl-β-D-Galactoside
ORF	Open Reading Frame
OXPHOS	Oxidative phosphorylation
PABP	Poly A Binding Protein
PCE	Post-Transcriptional Control Elements
PCR	Polymerase Chain Reaction
PIC	pre-initiation complex
PIPE	Protein-Protein Interaction Prediction Engine
PKC	Protein Kinase C
PPI	Protein-Protein Interaction
PSA	Phenotypic Suppression Array

qRT-PCR	Quantitative RT-PCR
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
SC	Synthetic Complete media
SD	Shine-Dalgarno
SDL	Synthetic Dosage Lethality
SDS-PAGE	Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis
SGA	Synthetic Genetic Array
SGD	Saccharomyces genome database
TC	Ternary Complex
mRNP	ribonucleoprotein mRNA
mTOR	mammalian Target of Rapamycin
tRNA	transfer RNA
URA	Uracil
UTR	Untranslated Region

WT	Wild Type
X-gal	bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YPD	Yeast extracts peptone dextrose media
4E-BPs	eIF4E binding protein
5’ Cap	7-Methylguanosine (m7G)

1. Introduction

1.1 Systems Biology

1.1.1 Functional Analysis in Systems Biology

Treatment for many diseases that so far have proven to be "incurable" including different types of cancer, diabetes, certain heart conditions and Parkinson's and Parkinson's-like diseases has formed a major focus of cell biology research. Numerous genes have been discovered linked to the onset of these complex diseases [1,2] and much has been learned. To date, however, a feasible approach to untangle the problem of complex diseases has remained ambiguous. It is generally accepted that complex diseases are not linked to phenotypic consequences of one or a few genes, but rather a cascade of circumstances in a larger and dynamic system in association with a number of interconnected genes and factors that influence the onset and progression of these diseases. These genes are not connected to each other by a simple network but rather an interactive and dynamic web of interactions and feedback loops that are needed to mediate their activities as a system [1,3]. Therefore, there is a need to investigate complex diseases in the context of such interconnected networks of interactions and as dynamic biological systems.

To better understand the balance between tens of thousands of compounds that work in harmony regulated by genetic information in the cell [2] it is inevitable to investigate a biological cell as an integrated unit [4].

Studying the behavior of molecules and dynamic interactions of genes and pathways at a system-level is the first step of the journey to understand how single cells, tissues, and organs interact with one another, respond to internal and external stimuli and how the living system functions. In addition, systems biology allows us to investigate the function of genes or proteins in multiple pathways at the same time [5]. The idea of a single function for a protein has faded over the years. Now we know that a single protein can function in multiple pathways, can move from an organelle to another to participate in a completely different process and interact with different sets of proteins to perform various functions. For example, eIF4E, one of the important factors in the initiation of the eukaryotic protein synthesis pathway, is not only crucial to translation but also can participate in transporting mRNAs from the nucleus to cytoplasm [6]. Systems biology can help us better investigate the various functions of proteins in a single cell or even in different tissues of a multicellular organism. Once we have a more clear understanding of the interaction networks for a target protein, we would be able to better realize the activity of that protein in a different context such as the onset and progression of different diseases [3].

Systems biology received considerable attention after the development of certain tools including whole genome sequencing, DNA Microarray analysis, high throughput protein identification using Mass Spectrometry, and various proteomics approaches among others [7]. These tools allowed scientists to observe the structure and dynamic changes in the entire system at the same time [4]. Network models derived from such observations and analysis, helped in simplification of the connected function of proteins in the systems [3]. As Kitano explains in his review [4], there are four main approaches to understand the

connection of genes and proteins at system-level, the first approach is to investigate the genetic network of genes and pathways. The second is studying the system's dynamics over time under different conditions. Third, to see how the control mechanisms work to maintain the state of the cell and prevent any dysregulation. And forth is designing an acceptable model based on known facts and simulations[4]. Shown in Figure 1.1, a proposed algorithm for designing a systems-level approach is illustrated.

A big challenge in molecular systems biology is to identify the relevant information from a sea of data, much of which often contains a high occurrence of background noise and artifacts. An inherent disadvantage of large scale experiments and high throughout screens is that they generally suffer from high rates of false positives and negatives [8–13]. Consequently, analyzing data is a crucial step that should be taken into consideration really carefully [14]. The development of improved methods has significantly improved the quality of generated data [15–19]. Additionally, advanced bioinformatics tools have been developed to screen through large biological data in order to better separate false positives [9,20,21].

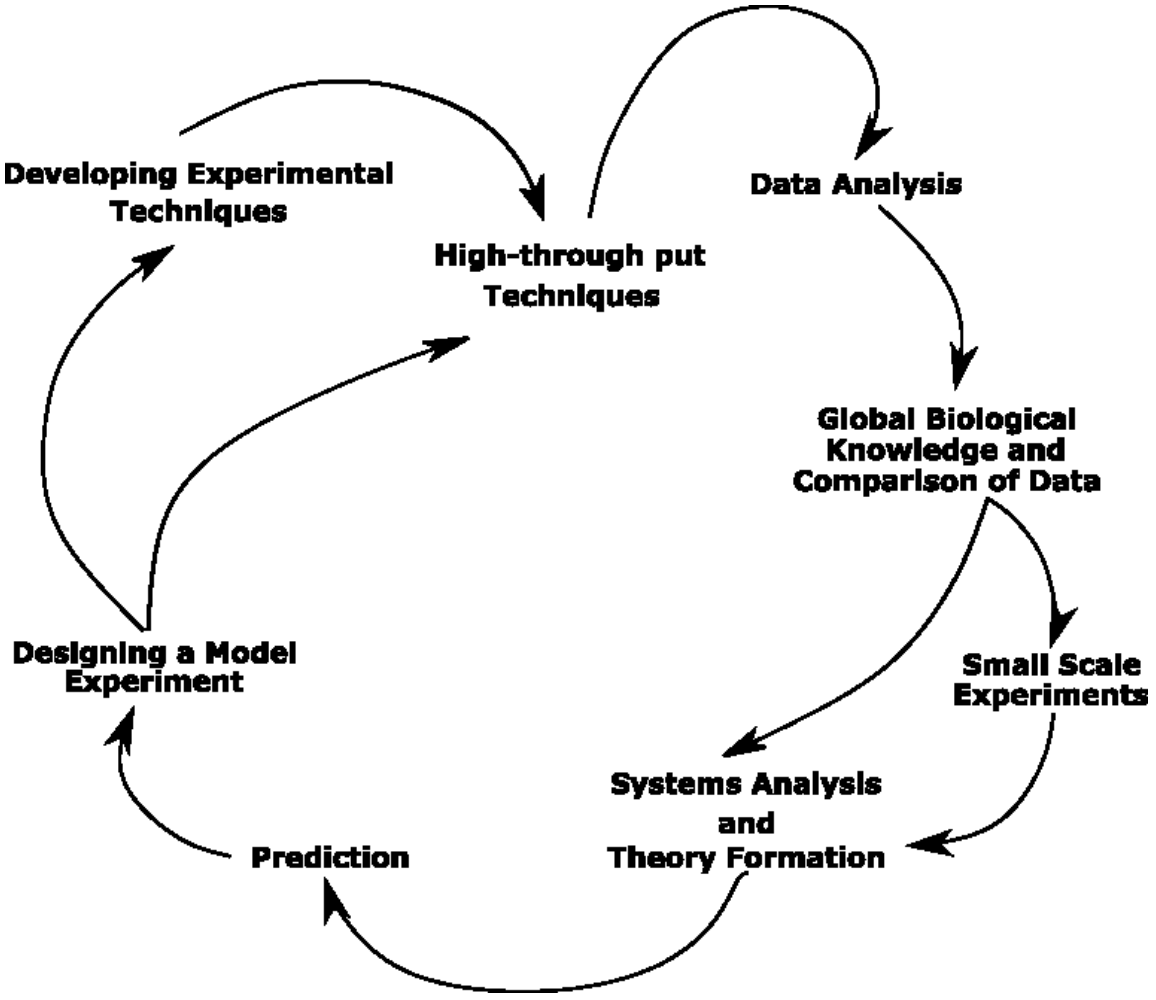


Figure 1.1 Experimental approaches in systems biology. The research starts by studying the literature and collecting data, analysis of the information and making predictions based on the available data. The next step is to design an experiment to test the hypothesis through high-throughput experiments and developing more efficient techniques to collect new data. The last step is to analyze the collected data from the experiment and compare it to the literature to confirm the hypotheses. Small scale (follow up) experiments can provide further support for the outcome of large scale experiments.

1.1.2 Functional Genomics

High-throughput techniques and specific small-scale experiments allow us to collect biological data for various biochemical networks within the cell, whereas mathematical modeling and bioinformatics tools are crucial for analyzing and making sense of the result

[2,21,22]. Visualization of data constitutes a different challenge for which a number of bioinformatics tools have been developed [23–25]. In these tools, genes/proteins are often represented as nodes and the networks of genes and proteins are represented as edges, which often constitute physical or genetic interactions. Much of this data and elucidated networks could be used to further investigate gene functions in a systematic way. Functional genomics aims to investigate the novel function of genes and proteins using high-throughput techniques and genome-wide data analysis [26–29].

The baker's yeast, *Saccharomyces cerevisiae* is a single-cell eukaryotic model organism that has been broadly used, not only to investigate various cell processes and pathways in molecular biology investigations [28,30,31] but also as a model organism of choice in functional genomics, proteomics, and evolutionary genetics [32–35]. This model organism has a short life cycle, its genomic manipulation is fairly easy, possesses both haploid and diploid life cycles (aids in a number of high-throughput techniques), is the first eukaryote to have its genome sequenced, and genomics and proteomics annotations are available through different databases (SGD; www.yeastgenome.org, and https://yeast.biocyc.org/ [36]). Yeast is also a valuable asset for human-related genetic studies; there is a 30% homology between human and yeast genome, 87% of yeast protein domains are present in human proteome and nearly 50% of the genes involved in human heritable diseases have a homologous counterpart in yeast [36–38]. In a recent study, 47% of the yeast genes were replaced by their human orthologues and strains showed recovery from their deletion mutants; 53% of the genes involved in the translation pathway showed to be completely replaceable in yeast by human orthologs [39]. This is despite the fact that there

were notable differences in gene sequences, splicing and protein assembly between orthologues genes [39]. Mercatanti et al (2017) analyzed 8078 missense mutations found in 31 cancer-related genes with homology in yeast, covering more than 50% of the missense variants causing cancer, making yeast-based functional assays a valuable source for cancer research [38]. This is in addition to the capability of evaluating yeast paralogues for therapeutic reasons.

Naturally, in the early days of systems biology, yeast was used as a tool for the development of high-throughput methods and large scale screenings [36]. To date, it continues to serve as a reliable resource for developing new and improved methods to systematically study a cell [40–42]. Many of these tools were developed to study protein-protein interactions (PPI) [16,19,43] whereas others are used to identify genetic interactions (GIs) [18,44,45]. Both PPI and GI data are heavily used to study functional genomics in order to assign novel functions to different genes under various conditions [27,31,34]. They are utilized in form of a "guilt-by-association" concept, where interacting proteins are thought to be functionally related [46]. For example, Krogan et al (2003) used PPI data to connect the Paf1 complex, associated with elongating polymerase activity, to histone methylation providing evidence for a connection between chromatin remodeling and transcription elongation [32]. Similarly, Butland et al (2007) connected the activity of bacterial Dead1 protein to translation initiation [47]. PPI data analysis continues to be an important tool for studying novel gene functions. For example, Omidi et al (2018) used PPI investigations to connect the activity of and previously uncharacterized proteins to the process of no-homologous end joining in yeast [27]. Similarly, Babu et al (2018) using a

global PPI investigation approach, identified a number of proteins with novel roles in envelope protein complexes in bacteria [48].

Similarly, GIs have been used extensively to elucidate novel gene functions. The following section focuses on the use of GIs in functional genomics.

1.1.3 Genetic interaction

As mentioned earlier, yeast has practical features that enables large-scale screening and investigate protein and genetic interactions in different pathways. System-level analysis of yeast with *Saccharomyces* Genome Project in 1996, identified nearly 6000 open reading frames (ORFs) [49]. This was followed by the Genome Deletion Project that lead to the development of yeast non-essential Gene Deletion Array, also known as loss-of-function in which more than 96% of the yeast genes were each successfully deleted providing scientist with an important tool to study gene functions [50]. Gene deletion mutant library has been used for genome-wide investigation of pathways and gene functions in a living cell and under different conditions like stress or target pathway of different compounds [31,33,34,44,51].

The functional relationship between genes and pathways can be described based on genetic interactions [52]. GI is loosely defined when the phenotype of a double-mutant is unexpectedly different from that of single mutants [53] (Figure 1.2); it helps to explain a higher level of association between the functions of two genes in parallel pathways (epistasis) or in the same essential pathway [53]. Negative GI is assumed when an

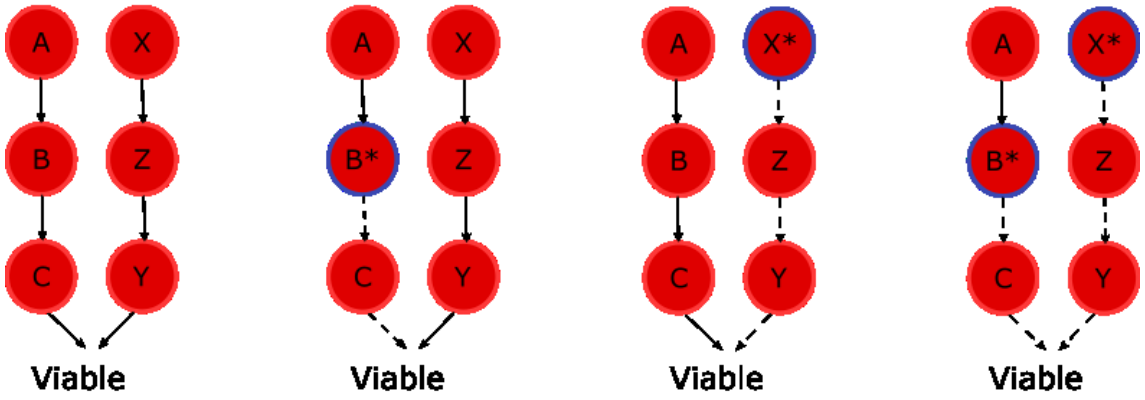
aggravating phenotype is observed, for example the fitness of cells measured by the size of the colony formed becomes significantly smaller than expected colony size of single mutants (Figure 1.2B). In this case, GIs are said to have synthetic sick phenotype or, in extreme cases, synthetic lethality. Similarly, positive genetic interaction refers to cases where double mutants have alleviating, or in this example bigger colonies, than expected from the phenotype of single mutants (Figure 1.2D) [30,31,53].

Yeast is considered an ideal tool for investigating systematic GIs because 80% of its genes are non-essential, and colony size can be used as a measure of fitness. Availability of two mating type makes systematic generation of double mutants a relatively easy task [7]. High throughput generation of double mutants in yeast is possible through a technique called Synthetic Genetic Array (SGA) analysis. In brief, a single gene deletion marked with a selection marker in alpha mating type is crossed to the array of single gene deletion mutants of a-mating type carrying a different selection marker. This procedure is made possible using a replica-pinning approach where floating pins, usually in a 384 format, are used to deliver yeast colonies from one plate to another [24,53]. After a few rounds of selections that includes sporulation, the progeny of a-mating type that carry double gene deletions is selected. The fitness of double gene deletion mutants is then analyzed using their colony size. The bigger the colony the higher the fitness. The size of the colonies can be automatically measured and analyzed using various software's [24,54,55].

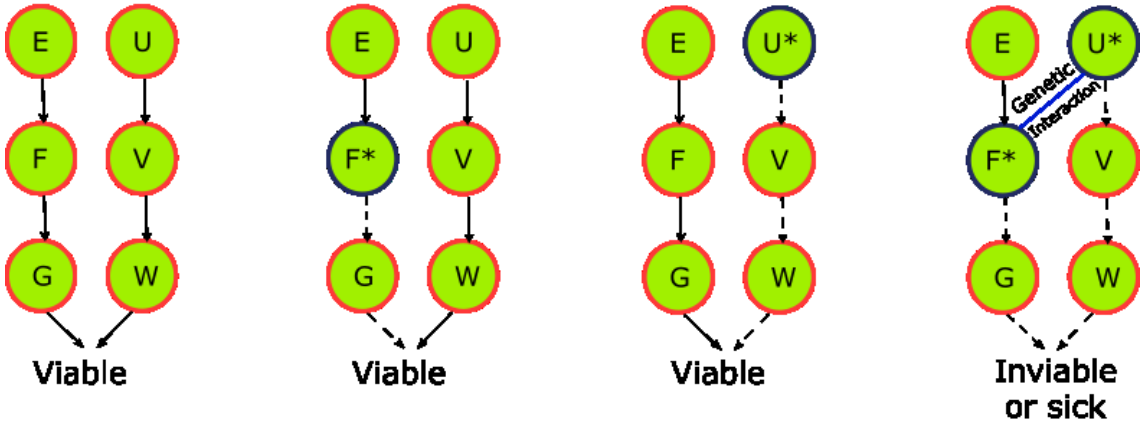
A second type of negative GI is termed dosage lethality. In this case, the overexpression of one gene results in an unexpected aggravating phenotype in a gene deletion background (Figure 1.2E) [56]. The high throughput method to study such interactions in

yeast is made possible by a modified SGA approach. In this case, after mating and sporulation steps, the “a” mating type progeny containing a non-essential gene deletion that carries an overexpression plasmid for the query gene is selected. As above, colony size can be used as a measure of fitness.

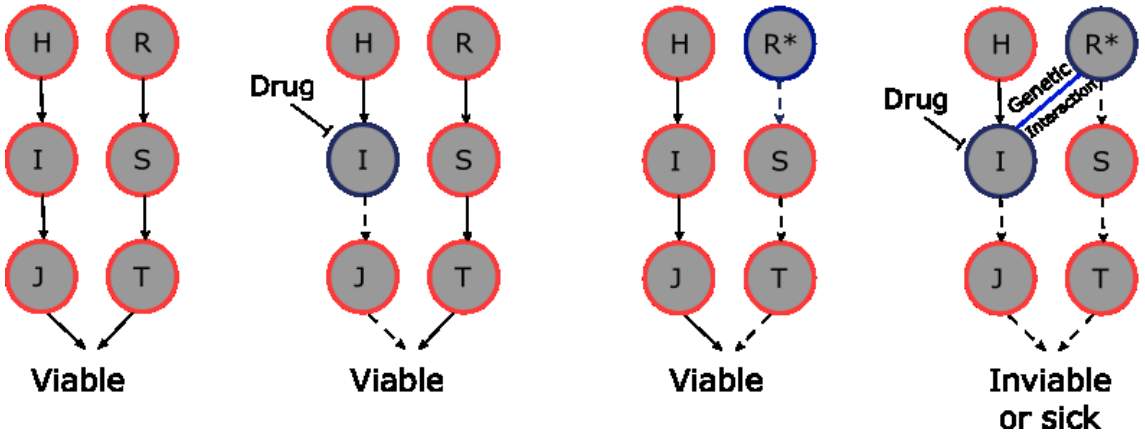
A) Non-interacting pathways



B) Genetic Interaction pathway



C) Chemical Genetic Interaction



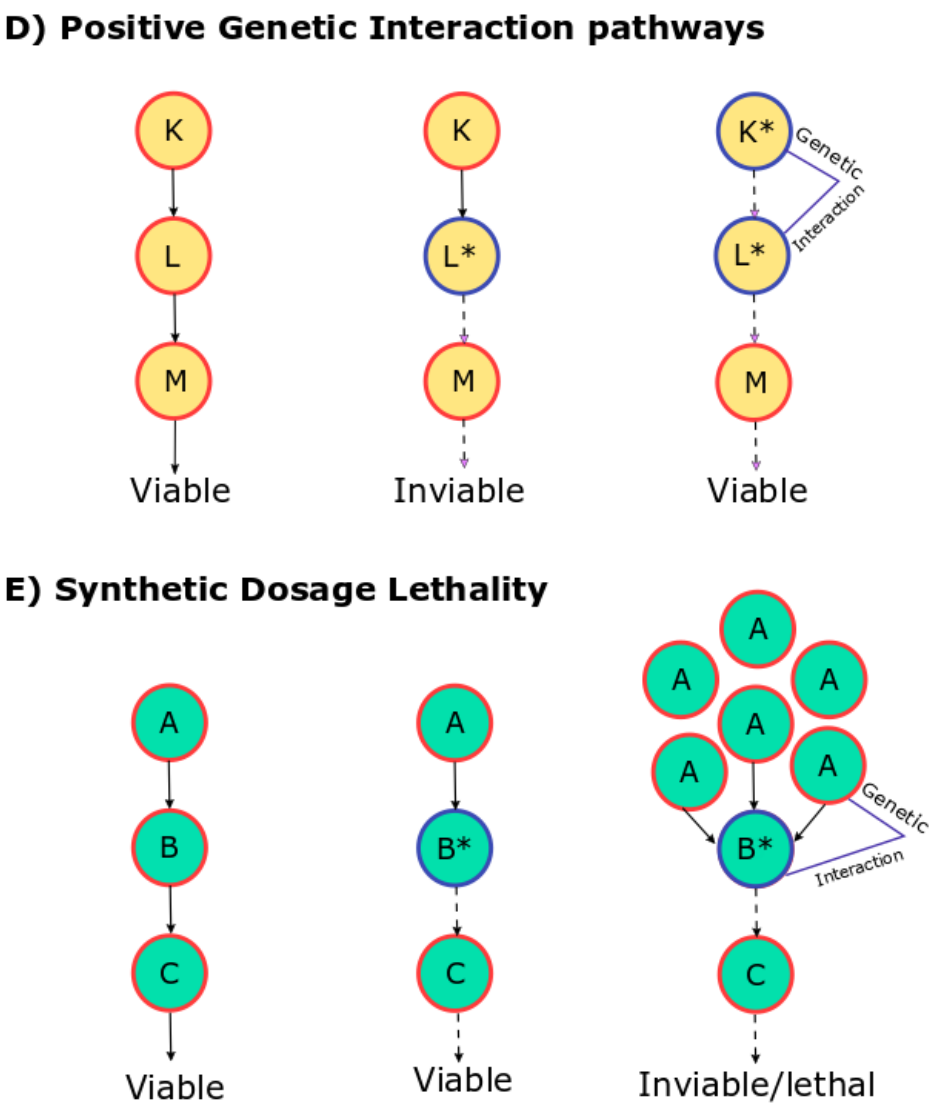


Figure 1.2. A schematic representation of genetic interactions (GI) and non-interacting pathways. A) The two pathways are independent of each other. Double mutants are viable. B) Two pathways are parallel and compensating. Double gene deletion leads to sickness or lethality of the cell, (negative GI). C) A gene deletion mutation in (B) is replaced by a chemical that might deactivate the gene product. This form of GI is called chemical genetic interaction. D) In positive GI, the deletion of a second gene reverts the sick/inviable phenotype of the first mutation. The second gene is often upstream of the first in the same pathway. E) Dosage lethality interaction is seen when over-expression of one gene in a single mutant background proves to be lethal. "*" means the gene is deleted.

Once the GI profile(s) for a target gene is determined, potential gene functions may be assigned. For example, it is expected that genes that are associated with protein biosynthesis would form GIs with protein biosynthesis genes [31,34] whereas those associated with DNA damage repair would interact among themselves [28,57]. Generally, it is accepted that genes that function on parallel compensating pathways can often form negative GI when both are deleted. Dosage lethal genes, on the other hand, are thought to be on the pathway where the overproduction of a product made by one gene may prove to be lethal to the cell.

Another form of GI can be studied under the treatment of chemicals, serving as modulators of genes. This area of research is often called Chemical Genomics [7,58] (Figure 1.2C). One way to study chemical genomics is using a chemical as a second mutation in a cell. In this case, a gene deletion mutant is used as the first alteration and the mode of action of a chemical is used as the second alteration. The chemicals with known mode of actions can be used to predict the function of an interacting gene in a parallel pathway [33,59–62]. Sub-inhibitory concentration of chemicals may also be used to further study GIs [63–65]. In this case treatment of double-mutants under the sub-inhibitory concentration of the chemical can generate new GIs forming conditional GIs [7,33]. In general conditional genetic analysis (under stress or drug), or conditional SGA, allows scientists to identify genes that are activated or induced under specific conditions revealing the novel function of genes and pathways. Based on the fact that certain pathways and genes are only

expressed under specific stimuli, conditional SGA can be a valuable method to study novel gene functions [33,34,66].

1.2 Translation

1.2.1 Translation pathway and the significance of studying it.

Protein synthesis is a highly conserved process that makes polypeptide chains from genetic codons transcribed on mRNA templates [67]. Proteins are important for almost all cellular reactions, structural molecules, in transportation, cell signaling and other important mechanisms [68]. Ribosomes are complex macromolecules that constitute the "machinery" of the translation process. In addition to their role in translation, ribosomes are also involved in engaging kinases and consequently signaling pathways [69]. Cell signaling pathways affect translation regulation by modifying the activity of translational factors through phosphorylation by the target of rapamycin (TOR) and mitogen-activated protein kinase (MAPK), among others [6,70]. Protein synthesis consumes up to 50% of the cell energy and in yeast equivalent to 30-40% of the cytoplasm volume is occupied by ribosomes (approximately 200,000 ribosomes) during growth stages [68]. Ribosomes are considered the core components of protein synthesis, consisting of two main subunits, small subunit with a crucial role in decoding the genetic code, and large subunit that contributes to the formation of polypeptide bonds. Ribosomal subunits are composed of proteins and rRNAs; rRNAs form the main enzymatic and structural component of the complex [71,72].

The translation process in eukaryotes and prokaryotes includes three major steps: initiation, elongation, and termination. One of the main differences is that in prokaryotes protein synthesis and transcription are taking place in the cytoplasm and can be coupled together at the same time, whereas in eukaryotes transcription is taking place in the nucleus and translation is in the cytoplasm after maturation of mRNA through splicing, addition of 5' cap and poly(A) tail and transport out of nucleus [67].

Translation initiation in prokaryotes starts with binding of the small subunit of ribosome (30S) consisting of the 16S rRNA and 21 proteins, to the unstructured region on mRNA called Shine-Dalgarno (SD), complementary to 16S rRNA sequence 5' to the start codon [68,72,73]. The recognition of the translation initiation site in prokaryotes is generally based on an RNA-RNA interaction [67]. Then large ribosomal subunit (50S), consisting of 5S and 23S rRNAs and 33 proteins, binds to small subunit to form 70S initiation complex (IC) to enter elongation step in prokaryotes [72].

In elongation step, peptidyl transferase activity of large ribosomal subunit synthesizes peptide bonds between corresponding amino acids, based on triplet nucleotide codons of mRNA. This is done with the help of elongation factors and other elements [67,68]. When the termination site is reached, at stop codons, instead of amino acid incorporation, termination factors enter the ribosome and induce dissociation of the polypeptide chain and ribosomal subunits from mRNA [67].

The translation procedure in eukaryotes follows the same overall concept with a bit of variation throughout the process. The first thing to notice is the larger size of the

ribosomal subunits, the 40S small subunit that consists of 18S rRNA, and the 60S large subunit composed of 5S, 5.8S and 25S rRNAs, 80 proteins (79 in yeast), that together form the 80S ribosomal complex [67,69,71,72]. Recognition of mRNA and initiation step differs in the eukaryotic system compared to prokaryotes. Eukaryotic mRNAs undergo a series of modifications to form mature mRNAs before translation can take place. In general mRNA splicing, addition of 7-Methylguanosine (m^7G) at 5' end of mRNA (5' cap) and poly(A) tail at their 3' end [67] lead to the maturation of mRNAs. In brief, the small subunit of the ribosome with the help of initiation factors recognizes the 5' cap and brings the initiator methionyl-$tRNA_i$ to the pre-initiation complex (PIC). The process starts by scanning for the correct AUG start codon [67].

Translation initiation is regulated by IF1, IF2 and IF3 in prokaryotes and eIF1, eIF1A, eIF2 (subunits α, β and ϒ), eIF2A, eIF3 (subunits a-m in mammals and a, b, c, g, h and j in yeast), eIF4F (subunits 4A, 4E and 4G), eIF4H, eIF4B, eIF5, eIF5A, eIF5B and eIF6, among others, in eukaryotes. Not only are these factors are important in starting the translation process but they are also important in controlling translation efficiency and are known to be controlled by various regulatory pathways [73–76]. Elongation step is highly conserved between eukaryotes and prokaryotes and termination, although relatively conserved, is promoted by different proteins.

Although much has been learned about these steps, there appears to remain other proteins and complexes affecting translation efficiency, and especially the regulation of translation needs to be further investigated. To give an example, the role of eIF2A is known to induce binding of Met-$tRNA_i$ to 40S ribosomal subunit, but Dmitriev and et al.,

proposed the role of another initiation factor, eIF2D to encourage the binding of tRNA to ribosomal P-site in absence of GTP [77]. Or the clear function of eIF5A was known to be essential for translation initiation, but recently it was proposed its role in translation elongation and termination [78]. Also, recently it has been shown that translation regulation is controlled not only by translation factors but also by ribosomal proteins, known to have structural features involved in translation regulation. Protein components of the ribosome have been shown to engage in transcription, DNA repair and life span in yeast [79]. The role of eIF4A, the main RNA helicase in translation is not clear either. Studies have shown that probably different types of RNA helicase are regulating different mRNAs in different tissues with or without correlation with eIF4A [76,80]. The list of proteins affecting both prokaryotic [81–84] and eukaryotic translation [9,34] continues to grow. There is a lot more to be understood about this process and its regulation in addition to its cross-talk with other processes. The main focus of the current thesis is to investigate new RNA helicases and associated factors, involved in this pathway, specifically those associated with the translation of highly structured mRNAs.

In addition to the central role of translation in the process of gene expression, a number of diseases have been linked to protein biosynthesis dysfunction, making translation pathway a major target for treatment [6]. Not only the translation machinery but also the dysregulation of genes involved in translational control has been shown to affect oncogenic pathways and consequently lead to tumor development [6,85]. For example, eIF4E is considered as one of the important targets of cell signaling pathways to induce growth and proliferation. It is thought that eIF4E malfunction is connected to

abnormalities in cell growth as seen in various cancers including prostate, breast, skin, lung, colorectal, bladder and cervical cancers [6,86]. eIF4E has also recently been connected to autism spectrum disorder [87]. eIF4A is overexpressed in melanoma cells where eIF3f is excised (subunit of eIF3) [86,88]. eIF3 is overexpressed in breast cancer and eIF4E overexpression is linked to lung carcinoma [85]. eIF4A inhibition (with Hippuristanol) is shown to delay Alzheimer's disease progression in early stages of recognition when cells are under oxidative stress [89]. Also, the inhibition of eIF4A can selectively reduce the translation rate of genes involved in the progression of Alzheimer's disease including A-beta (Amyloid β peptide) and tau (microtubule-associated protein) genes that happen to have highly structured 5'-UTRs but not the housekeeping genes [89]. The connection of translation to various diseases provides us with additional reasons for investigating this process and its regulations.

1.2.2 Translation Initiation step

The initiation step of translation is the most regulated step of the pathway [6,76,90]. In the pre-initiation of cap-dependent eukaryotic translation, Met-tRNA$_i$ promoted by eIF1, with eukaryotic initiation factor 2 (eIF2), and GTP forms a complex called ternary complex (TC). TC binds to the small subunit of ribosome (40S), making 43S PIC that includes other initiation factors eIF1, eIF1A, eIF5 and eIF3 to scan for the start codon [74,76]. eIF1 and eIF1A are involved in the opening of mRNA and tRNA binding grooves for scanning and detecting the start codon as well as preventing the association of large subunit at this stage [72]. The role of eIF6 is not well understood but there are suggestions that it might prevent the early recruitment of large subunit to the PIC [72]. Before scanning, PIC binds to 5' cap of

mRNA with the help of eIF3, eIF4H, eIF4B, and eIF4F, that consists of eIF4E as cap-binding protein, eIF4A an RNA helicase and eIF4G as scaffolding protein to bind the complex to poly(A) binding protein (PABP) at 3' end of mRNA (Figure 1.3) [75,90]. eIF4A, is not the only helicase enzyme involved in this complex, but it is suggested that other enzymes like Ded1 may also bind to this complex and function as helicases [91]. Binding of PIC to PABP makes a stable circular ribonucleoprotein mRNA (mRNP) to facilitate the restarting of translation on the same mRNA [76]. mRNP and eIF3 allow the association of 43S complex and helicase activity of eIF4A helps unwind the secondary structure with the help of eIF4B to stimulate its ATP hydrolysis and start scanning [76,92]. eIF3 is a large complex of 13 different proteins in mammals and six in yeast that binds to mRNA and stimulates PIC assembly for proper scanning [93]. eIF4F complex, plays crucial roles in recruiting 43S subunit to mRNA [90]. For highly structured untranslated regions (UTRs) mRNAs, the need for eIF4A and eIF4F is higher [76,94]. Base-pairing of Met-tRNA$_i$ anticodon and AUG in the P site of small ribosomal subunit induces the release of eIF2 and GTP-activation of eIF5, the release of eIF2,GDP and other eIFs including eIF1 of the complex and consequently association of 60S ribosomal subunit by eIF5 and formation of 80S IC followed by the entry of machinery into elongation step [76,90,95].

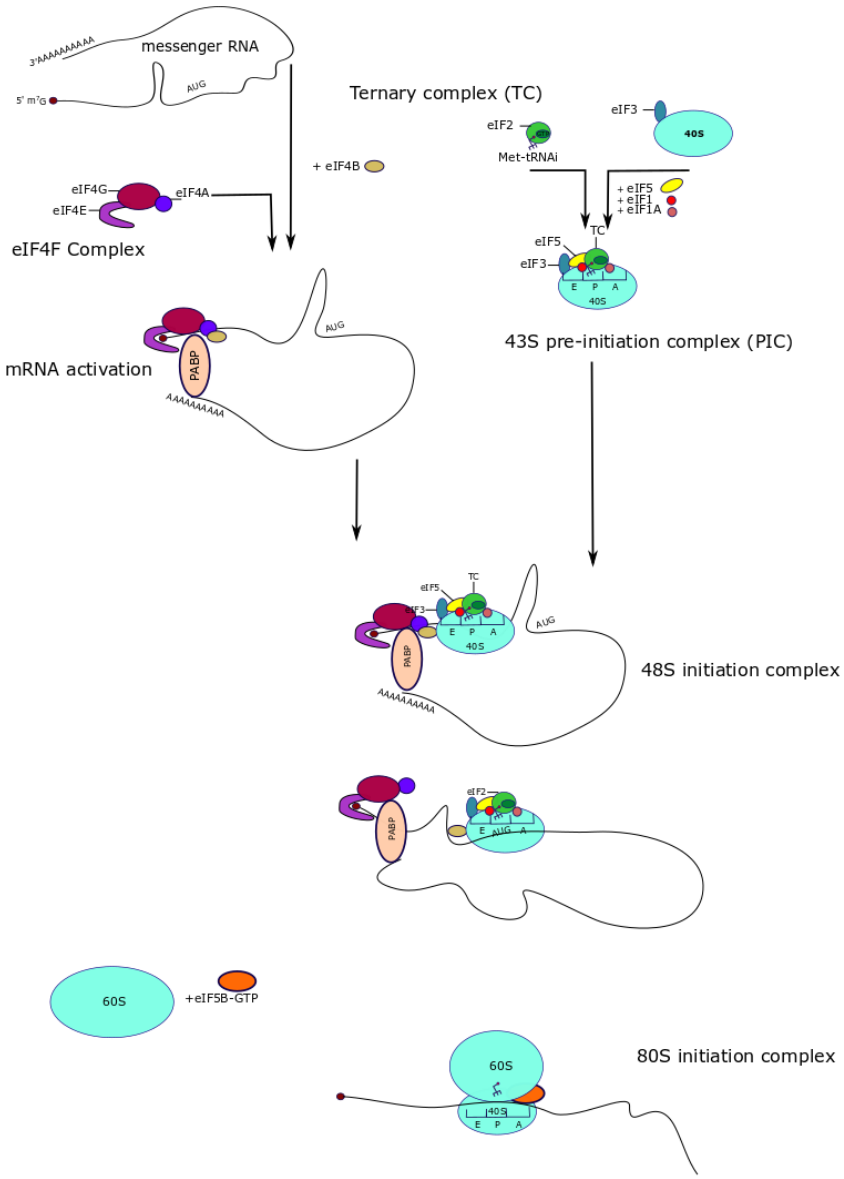


Figure 1.3. A schematic illustration of the eukaryotic translation pre-initiation step. The pre-initiation step starts with mRNA activation when the eIF4F complex binds to 5' cap of mRNA. Then TC carrying the initiator methionine amino acid binds to the 40S subunit of the ribosome along with other initiation factors to form PIC. The recruitment of PIC on activated mRNA forms 48S IC, ready for scanning the mRNA in search of start codon. When AUG is detected eIF2 is induced to dissociate from the IC along with other translation initiation factors. This will allow the 60S ribosomal subunit to bind to the 40S subunit with the help of eIF5B to form 80S IC ready to move to the elongation step.

In the prokaryotic system, the initiation step is regulated through three main factors, IF1, IF2, and IF3 to find the right start codon and recruit initiator tRNA into the P-site of small ribosomal subunit [72].

1.2.3 RNA helicases

RNA helicases are not only involved in unwinding of secondary structure (also known as hairpins or sometimes inhibitory structures) on mRNAs during translation but also are involved in transcription, mRNA splicing, mRNA export, mRNA storage, and mRNA decay pathway [91,96,97]. DEAD box helicases, a common family of conserved helicases that contain DEAD motif are involved in unwinding RNA hairpins, remodeling ribonucleoprotein complexes for better scanning and recruitment of macromolecules of translation machinery on mRNA, spliceosome complexes and mRNA nuclear export [98]. In fact, in many cases, it seems that the fate of the mRNA, to be translated, degraded or stored depends on different types of helicases [99].

eIF4A, the main helicase for translation initiation belongs to the DEAD-Box family of RNA helicases that have a highly conserved domain called RecA. This domain forms a stabile structure after binding to ATP and RNA, promoting the helicase activity [92]. eIF4A is an important component of the eIF4F complex in the cap recognition in proliferating cells. This fact makes helicases possible oncogene and potential targets of anti-proliferative drugs. Pateamine A, Hippuristanol and Silvestrol are the inhibitors of eIF4A discovered over the past decade to control translation initiation in growing cells [100].

eIf4A is generally thought to be the main RNA helicase in the translation pathway responsible for unwinding secondary structure of mRNA in the initiation step to allow scanning for start codon AUG. It has been shown that multiple molecules of eIF4A can be recruited to an individual mRNA in the PIC [76]. However, it is not clear if this factor (eIF4A) continues unwinding the mRNA along the way or does other helicases/proteins function or associate with eIF4A in the process. It is thought that in different body tissues, different helicases replace eIF4A in the IC [80].

DED1 and its close homolog DBP1 are the two of the reported DEAD-Box RNA helicases in yeast with homology to *DDX3* in mammals. *DED1* is involved in mRNA splicing and its depletions affect the splicing as well as the global translation rate [91]. It was recently observed that *DED1* methylation affect the regulation of specific mRNAs [101]. Its mammalian homolog, *DDX3* is also involved in transcription, mRNA export, and translation. Its role in translation has been connected to Internal Ribosome Entry Site (IRES) regulating cap-independent translation regulation [91]. More recent studies proposed the cap-dependent regulation of *DDX3* for specific mRNAs with structured 5'-UTRs happens to be close to 5' cap, suggesting their role in recognition of cap and cooperating with eIF4A to unwind and scan the transcript [98].

Another group of DEAD-Box helicases influence the translation of those mRNAs that contain post-transcriptional control elements (PCE) on their 5' termini [96]. It was observed that in some cases when the ribosome is stalled on mRNA and scanning has been interrupted, specific sequences in 5'-UTR region of mRNAs, which do not have IRES characteristic, will be recognized by this type of helicases. *DHX9* known as RNA helicase A

is an example of this group of helicases in humans [96]. It was proposed that RNA helicase A is important host factor for the proper translation of HIV-1 and a possible target for therapeutic purposes [98,102]. Helicases modify RNA-RNA and RNA-protein interaction to regulate translation. It remains a possibility that there are other proteins involved in the translation of specific types of mRNAs specifically for the ones with long structured 5'-UTRs. A major challenge in the coming years will be to identify the substrates that are selectively regulated by each RNA helicase at each step of gene expression. It has already been shown that some helicases, including *DDX3* in human, are regulating specific transcripts, containing PCE elements, which cannot be modulated by other enzymes like eIF4A [98] and that they seem to be important in ribosome recruitment before 43S subunit starts scanning by remodeling the mRNA [98].

Advances in the field of RNA structure analysis will improve our understanding of how RNA helicases are potentially linked to various RNA structures. It was observed that in specific types of cancer overexpression of eIF4A, upregulate translation of genes involved in cell proliferation and growth happens to have highly structured 5' UTRs [89]. It is also important to note that small molecules can be developed to target the enzymatic activity of RNA helicases that would not only be useful to dissect the cellular functions of these factors, but also might possess therapeutic potentials, as several RNA helicases are involved in a wide array of diseases.

In humans, *DHX29* is an important RNA helicase that can regulate translation initiation. It's malfunction or downregulation affects the efficiency of translation and impedes cancer cell growth *in vitro* [90] making it a suitable target for cancer therapy. Other helicases

like *DDX3* are shown to interact with eIF4A and other initiation factors. These interactions may explain the mechanism by which such helicases affect translation regulation [91]. The existence of other helicases that can affect translation implies that there may exist other undiscovered helicases or helicase-affecting elements that can also influence mRNA translation [91].

1.3 Translational control

1.3.1 Translation control in gene expression

Regulation of gene expression is taking place at different levels starting from chromatin remodeling, transcription, mRNA maturation, splicing, mRNA export, followed by translation and post-translational modifications. mRNA content does not necessarily reflect the level of translation [6]. Translation control is considered an important step in determining the rate of protein accumulation. Also, is one of the fastest responses to stress is thought to be at translation step, where the cell can quickly regulate protein synthesis to reduce/induce protein production in response to stimuli [103].

Signaling pathways are known to be important in affecting the activity of translation factors in response to internal and external stimuli [70]. Translation control is important in many cellular processes including cell metabolism and proliferation and is shown to be connected to certain diseases including cancer and ribosomopathies, a class of inherited diseases caused by a malfunction in the component of ribosome structure like dyskeratosis congenita [85]. It is important to investigate and understand translation control

and the corresponding upstream and downstream pathways to better realize the biology of the mentioned diseases [85] and discovering potential therapeutic targets to tackle different diseases [104].

As mentioned earlier, protein synthesis requires a great deal of cellular resources (energy). Naturally regulating translation at the initiation step, is the most cost-effective measure to regulate protein synthesis [68]. A number of key parameters for the regulation of eukaryotic translation initiation relies on certain components on mRNA including *cis*-regulatory regions on 5'-UTR and 3'-UTR of different mRNAs, 5' cap structure, the length of mRNA, secondary structure of 5'-UTR, the Kozak sequence around AUG, poly(A) tail, uORFs, and *trans*-acting elements in addition to the availability of initiation factors, regulatory proteins and microRNAs [95,105]. In prokaryotes, the regulation seems a bit simpler and depend on the length of mRNA, SD sequence, non-SD translation enhancers [106–108] and initiators [109], stability of secondary structure at both 5'- and 3'-UTRs, length of coding region among others [110].

Translational control happens either at a global level for most mRNAs, or it applies to specific mRNAs with specific features [68]. In both scenarios, the goal is to change protein production or induce translation of mRNAs important to cell function under different conditions. Four general elements are thought to determine the global rate of translation, the mRNA level, abundance of the ribosome, rate of elongation and the activity of translation machinery (for example, phosphorylation of translational components) [68,111]. For more specific regulation, there are two general approaches to control the initiation step, regulation of translation initiation factors and utilizing *cis*-regulatory elements on

mRNA, modulated by proteins or small RNAs [112]. At least 12 initiation factors are directly involved in the control of translation initiation step and most of them are targets of translation regulation [76]. Initiation factors, with mRNA binding features, can be phosphorylated, affecting translation and growth of the cell. eIF2, eIF4E, eIF4G, eIF4B and eIF3 (eIF4A in plants) [68] are mostly regulated by mTOR and MAPK signaling pathways [70]. eIF4E in the eIF4F complex plays an important role in cap-dependent translation initiation where it competes with eIF4E binding protein (4E-BPs), a substrate of mTOR pathway in binding to 5' cap [70,113]. S6Ks form another group of translational repressors of mTOR pathway that regulate ribosomal protein S6, eIF4B and programmed cell death 4 protein (PDCD4) [70,114]. mTOR pathway not only has a negative regulatory effect on translation but also by phosphorylating eIF4B and eIF4H, it can improve the helicase activity of eIF4A. When it binds to PDCD4, eIF4A loses its affinity to eIF4G causing reduced cap-dependent translation [114]. The second regulation approach relies on 5'-UTR features of mRNAs that are important for both the recruitment of translation initiation factors and the rate of translation [115]. The most common *cis*-regulatory elements are 5' cap, hairpin and IRES structures, upstream open reading frames (uORF) and poly(A) tails [112,116]. uORFs are an important translation control in response to stress. It is thought that this mode of translation regulation affects 40% of all mammalian mRNAs [117]. Under certain stress conditions, uORFs are translated and the main ORF reading (the downstream one) is inhibited mostly through non-canonical initiation from uORF start codon and termination and dissociation of translation machinery before it reaches the main ORF [117,118]. Recently it was discovered that apart from uORFs role in inhibiting the translation of the main ORF,

in some cases they can recruit translation initiation factors more efficiently on mRNA and hence promote the translation of the main ORF. In this manner, in response to stress, uORFs can both downregulate or upregulate the translation of certain genes [117,119].

In contrast to the canonical mode of translation where the machinery recognizes the 5' cap and starts scanning for start codon, using alternative strategies, 5%-10% of mRNAs can be recognized directly by the 40S subunit of the ribosome. Some of these mRNAs contain specific structural elements called Internal Ribosome Entry Sites (IRES). IRESs are most widely used by RNA viruses to take advantage of the host cell translation machinery when general translation might be compromised. Certain proteins involved in cell growth and proliferation as well as those involved in the regulation of apoptosis, have been reported to use IRES-mediated translation [74,120,121] when the canonical translation is inhibited.

Ribosome shunting [122], tethering [123], translation enhancers [124,125], TISU element for short 5'-UTR mRNAs [95,123] and poly(A) leader can also regulate translation [74].

An interesting form of translation regulation is seen on specific mRNAs with 5'-UTR that carry poly (A) regions. This mode of regulation is often observed when eIF3 and eIF4F are not readily available. These poly(A) regions are often found just before the initiation codons and they appear to attract the 40S ribosomal subunit to associate with mRNA and form an IC in an ATP-independent manner [74].

In prokaryotes, translation is most commonly regulated directly by blocking the accessibility of machinery to the initiation site [73]. The strength of the complementary base

pairing of the SD sequence with the 3' end of the 16S rRNA is an important feature of translation regulation. Similarly, the availability of enhancer sequences and other translation initiation sequences to base pair with different regions of 16S rRNA also seem to play a role in the initiation of translation [107,126,127]. mRNA-16S rRNA base pairing can be regulated by the structures within the mRNA or through mRNA-binding proteins [73]. Ribosome occupancy and density in the cell also play an important role in prokaryotic translation regulation [110].

Translation regulation in mitochondria resembles those of prokaryotes. The Mitochondrion is known to have its own genomic information (known as mitochondrial genome (mtDNA)). However, approximately 95% of the proteins involved in mitochondrial pathways have nuclear origins including those affiliated with oxidative phosphorylation (OXPHOS), which is the main pathway for producing cell energy [128]. These mRNAs are directed to the vicinity of mitochondria for translation [128–130] along with some tRNAs and other RNAs including rRNAs and lnRNAs [130]. Some tRNAs are interchangeable between cytoplasm and mitochondria and some are specific to mitochondria [130]. mRNA transport into mitochondria is thought to be based on sequential interaction of mRNA with directing enzymes like Eno2P and pre-LysRS in yeast [130]. For rRNA import, both sequential interaction and membrane proteins are crucial for proper transfer into mammalian mitochondria [130]. Some mitochondrial mRNAs like *COX2* have 5' upstream regulatory regions that promote their translation in yeast mitochondria [129]. Translation regulation in mitochondria is proposed to be under indirect regulation of cytosolic translation [128].

1.3.2 Translation control of mRNAs with structured 5'-UTRs

In eukaryotic cells, translation control can regulate cell development, growth, tumorigenesis, differentiation, cell proliferation and protein production [6,67,90,131]. One of the cells fastest response to stress and apoptosis relies on translation control at the initiation step [121]. Translation initiation is considered the main target for translation control [67,76,96,115]. Other mechanisms including frameshifting and ribosomal stalling during the elongation step, also play roles in protein synthesis regulation [73]. Dysregulation of translational control is connected to many diseases including different types of cancers [131]. Commonly, such dysregulation is observed at the initiation step where the 40S subunit is recruited to mRNAs [104]. eIF4F plays an important role in regulating the initiation step and its components have become important targets for therapeutic purposes [104]. eIF4E, one of the components of eIF4F is the cap-binding protein that under stress condition can be inhibited by phosphorylation of 4E-BP [6]. This regulation is thought to affect so-called eIF4E-sensitive subset of mRNAs that carry long and structured 5'-UTRs [70]. It was observed that mRNAs coding for proteins involved in proliferation, oncogenicity, and cell survival seem to carry such features and are regulated by the mentioned signaling pathway [70].

The structure of mRNAs in eukaryotes is an important feature of translation regulation. these features include the 5' cap structure, sequences flanking AUG start codon, the position of AUG from 5' end the secondary and tertiary structures within mRNA leader sequences [73]. In mRNAs with long 5'-UTRs the role of eIF1, eIF1A, *DED1*, eIF2B, eIF3, and eIF5 seems to be more important for proper scanning [93,131]. Some mRNAs have *cis*-acting

sequences, mostly at their 5'-UTRs, that attract different *trans*-acting proteins and small regulatory RNAs to influence translation regulation [105,112]. It has been shown that the free energies of secondary structure on 5'-UTR of mRNA does affect the translation rate [105]. Such structures are often subjected to unwinding by helicases under different conditions. mRNA structure is not only crucial in translation initiation rate but it can also affect elongation and termination [67]; it is thought that RNA helicases might be required to unwind different structures along the way [132]. The shape of mRNA has also been connected to stress granule assembly. This is specifically relevant to mRNAs with long 5'-UTRs that under stress conditions can be modified through cell cycle regulatory proteins such as *WHI3* [133].

In addition to eIF4A other DEAD/DExH-Box RNA helicase, including *DED1* and *DBP1* in yeast and *DDX3* and *DHX29* in mammalian, are involved in unwinding and scanning steps of initiation for mRNAs with highly structured 5'-UTRs, mostly involved in cell growth, proliferation, and morphogenesis [76,91,134]. Confocal microscopy analysis revealed the interaction of *DDX3* with eIF4G and PABP proteins under specific stress conditions; *DED1* was also observed to bind to eIF4G in yeast [135]. Recent evidence suggest that *DHX29*, *DHX9,* and *DDX3* in mammalians and *DED1* in yeast appear to be specific for scanning and unwinding of more complex mRNA structures [90,91,96,136] whereas the main role of eIF4A in eIF4F complex appears to be linked to attachment of 43S to mRNA and the scanning for moderately structured mRNAs [90,91,131,134,137]. Other helicases including *DDX3* bind to specific structures on 5'-UTR of specific mRNAs and regulate their translation by binding to eIF4A or independently like *Dbp5* helicase. [91].

mRNAs having long and highly structured 5'-UTRs requires a different mechanism for efficient translation [96]. This encouraged us to look and investigate more unknown helicases involved in recognition and unwinding of highly structured 5'-UTRs.

The sequence around the AUG start codon is a strong determinant of the translation rate, referred to kozak consensus sequence. If this sequence is not optimal the scanning ribosomes may bypass the first AUG codon and start translation from the second AUG. Such events can be regulated by the presence of a hairpin between the two AUGs. In this case, the balance between the production of the two ORFs, a longer and a shorter one, might be achieved by the formation of an mRNA structure between the two start codons and protein factors that influence the structure [73]. This phenomenon has been observed in plants, animal and yeast cells [138].

In prokaryotes, structural *cis*-acting components at 5'-UTR including riboswitches and RNA thermosensors may regulate translation [139]. Riboswitches respond to the binding of metabolites where thermosensors make secondary structures in response to temperature and regulate recruitment of the 30S ribosomal subunit [139]. In general translation initiation region of prokaryotic mRNAs contain an SD sequence. The length of this sequence, its distance to start codon and its involvement in secondary structures also play important roles in the regulation of translation. mRNAs with strong hairpin structures near the start site, generally require stronger SD sequence to efficiently attract the ribosome to the initiation site [73]. Polycistronic mRNAs often carry their own SD sequences upstream of different ORF. Therefore, the translation of each ORF is directly dependent on its corresponding SD sequence. In certain cases, the ribosome attached to the

upstream ORF is recruited to translate a downstream ORF. In such cases, the presence of a strong secondary structure in the translation region of the downstream ORF often inhibits the recruitment of the ribosomes from the upstream ORF [73]. Certain conditions like low/high temperatures or the presence of certain chemicals can induce specific folding of the mRNAs to inhibit or promote the translation of a specific gene [73]. mRNA folding in bacteria in response to environmental changes can also regulate mRNA stability. Certain repressor proteins can also bind to the upstream regions of the SD sequence and cause the mRNA to fold and prohibit the 30s from binding to mRNA [139]. Small *trans*-acting RNAs can also interfere with mRNA structures and regulate prokaryotic translation [73,139].

1.4 Focus

The process of protein synthesis is an essential measure for living cells. In addition to the need for studying translation as an essential process for cell survival, dysfunction in this process has been linked to important human conditions including complex diseases like cancer and diabetes highlighting the need to better understand this process. Although much has been learned over the years, there are a number of processes within translation that require more attention. The list of genes that control translation or influence its efficacy continues to increase.

One of the main objectives of this thesis is to discover and study novel genes that can affect translation in yeast. Specifically, I am interested in studying those genes that affect the translation of structured mRNAs. For this, I employ the yeast gene knockout collection as a tool to screen for those genes that when deleted, expression of a structured mRNA

reporter is reduced (Figure 1.4). For this purpose, a *β-galactosidase* reporter gene that carries a series of strong inhibitory structures on it 5'-UTR is used. Gene deletion mutants that were identified in our large scale screening are selected for follow up investigation to better elucidate their involvement in the translation of structured mRNA. In addition, by manipulating the UTRs of a reporter mRNA, I investigated the delivery of this mRNA into yeast mitochondria.

The specific objectives of this thesis are to:

1 Screen the yeast non-essential gene knockout collection for genes that when deleted reduce the expression of a highly structured reporter gene.

2 Investigate the activity of *YTA6* and *YBR096C* as novel genes that affect the translation of structured mRNAs (Chapter 2).

3 Study the involvement of *NAM7*, *PUS2,* and *RPL27B* in the translation of structured mRNAs (Chapter 3).

4 Examine the ability of the 3'-UTR of yeast *OXA1* gene in directing mRNAs into yeast mitochondria (Chapter 4).

5 Design and utilize other structured mRNAs to further screen the yeast non-essential gene knockout collection for additional genes that are required for the translation of structured mRNAs. (Chapter 5).

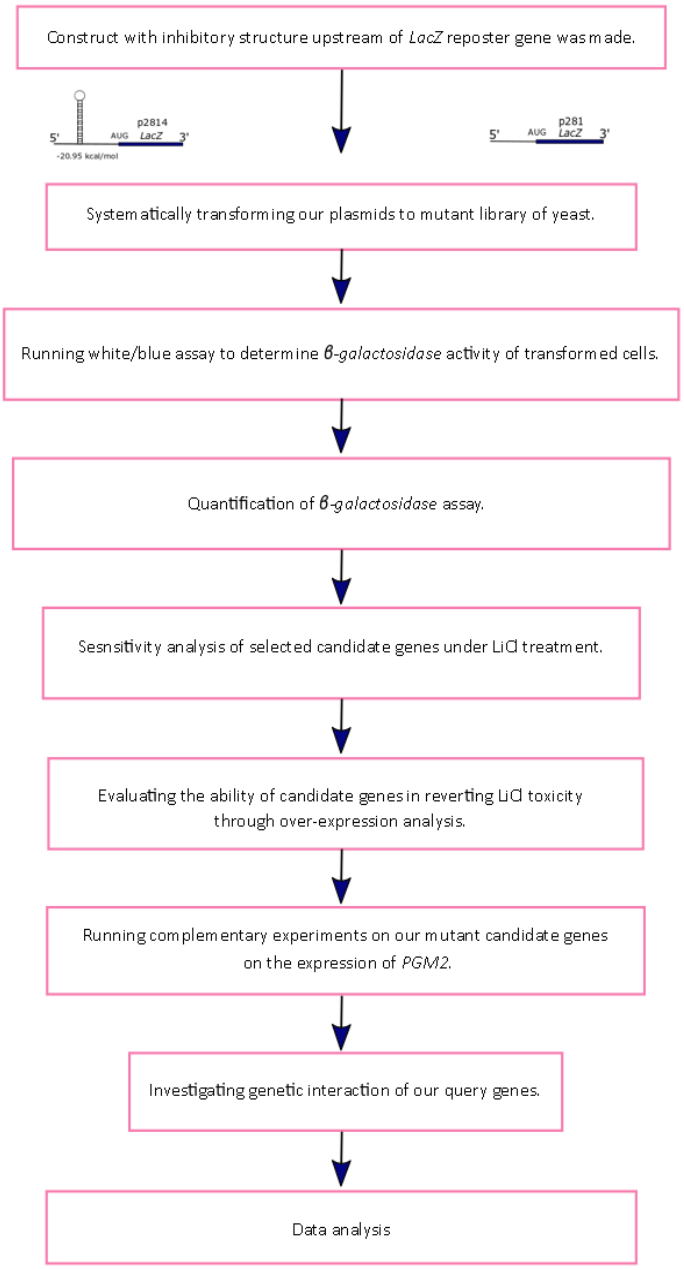


Figure 1.4. Diagram showing the step by step approach in this thesis. A construct with inhibitory structure upstream of *LacZ* cassette and a control plasmid were constructed [157]. Plasmids were transformed to deletion mutant library to determine in the absence of which candidate genes the cells were unable to produce *β-galactosidase*. 24 candidate genes were identified that did not show translation activity in large-scale lift assay (Blue/White assay). Translation activity was quantified using small scale *β-galactosidase* liquid assay to follow up with our query genes we ran sensitivity assay using treatment with LiCl, which has been shown to affect translation initiation linked to *TIF2* (eIF4A) helicase. It is reported that over-expression of eIF4A, the main helicase enzyme linked to translation initiation has been shown to revert LiCl sensitivity. We repeated this with over-expression of our query genes. Five candidate genes were selected. To further study the involvement of these target genes in the translation of structured mRNAs we ran a series of complementary assays including genetic interaction (GI) analysis.

2. Sensitivity of yeast to lithium chloride connects the activity of *YTA6* and *YPR096C* to translation of structured mRNAs

2.1 Abstract

Lithium Chloride (LiCl) toxicity, mode of action and cellular responses have been the subject of active investigations over the past decades. In yeast, LiCl treatment is reported to reduce the activity and alters the expression of *PGM2,* a gene that encodes a phosphoglucomutase involved in sugar metabolism. Reduced activity of phosphoglucomutase in the presence of galactose causes an accumulation of glucose-1-p leading to a number of phenotypes including growth defect. In the current study, we identify two understudied genes, *YTA6* and *YPR096C* that when deleted increase cell sensitivity to LiCl treatment in yeast. We further show that *YTA6* and *YPR096C* exert their activities by influencing *PGM2* at the level of translation.

2.2 Introduction

Dysregulation of signaling pathways in the brain is thought to be the main cause of bipolar disorder (BD) [140]. LiCl has remained an important treatment option for BD for more than ten decades [141,142]. It has been prescribed to prevent both new depressive and manic episodes and is known to be the only compound to have anti-suicidal effects in BD patients [143].

When LiCl is used as a therapeutic agent, it is generally accepted that in the short term, it influences Protein Kinase C (PKC) and glycogen synthesis kinase-3 (GSK-3) signal transduction pathways. Long term exposure to LiCl modifies the expression of different

genes/pathways including PI/PKC signaling cascade, leading to alterations in the synaptic function of nerve cells [140,144–146]. Inducing autophagy, oxidative metabolism, apoptosis and affecting translation machinery are other pathways proposed to be influenced by LiCl intake [141,145]. LiCl has also been investigated as a treatment option for Alzheimer's disease which is caused by the aging of the nervous system [145,147]. Although much has been learned about the influence of LiCl, how it affects the cell at the molecular level and the mechanism(s) of its activity, as well as its side effects (secondary effects) require further investigations [140,141,147].

At the molecular level, the sensitivity of yeast cells to LiCl was previously described by changes in the level of expression and activity for *PGM2* that encodes a phosphoglucomutase [148,149]. Phosphoglucomutase is responsible for converting glucose-1-phosphate to glucose-6-phosphate. When galactose is used as the carbon source, inhibition of phosphoglucomutase by LiCl results in the accumulation of glucose-1-phosphate that in turn causes growth defects [150,151]. In the presence of glucose, LiCl reduces the levels of UDP-glucose and disrupts the associated pathways. It has also been suggested that LiCl may inhibit RNA processing enzymes [152,153]. Also, it is reported that under LiCl stress, there seems to be a rapid loss of ribosomal protein gene (RBG) pre-mRNAs and a decrease in a number of mature mRNAs in the cytoplasm[153]. In addition, it is possible that LiCl may inhibit the initial steps of the protein synthesis pathway. It is thought that LiCl may disrupt the association of translation initiation factor eIF4A RNA helicase to the yeast translation machinery [148] impairing translation initiation. Deletion of

TIF2 that codes for the eIF4A helicase increased yeast sensitivity to LiCl. Over-expression of eIF4A helicase reverted the translational inhibition caused by LiCl [148].

In the current study, we observed that the deletion of two yeast genes, *YTA6* and *YPR096C* increased the sensitivity of yeast cells to LiCl. *YTA6* codes for a putative ATPase of the CDC48/PAS1/SEC18 (AAA) family of proteins and *YPR096C* codes for a protein of unknown function. Neither of the genes was previously linked to cell responses to LiCl. Our follow-up genetic investigations suggest that the involvement of *YTA6* and *YPR096C* in yeast LiCl sensitivity seems to be due to their influence on *PGM2* translation.

2.3 Materials and methods

2.3.1 Strains, plasmids, gene collections and cell and DNA manipulations

MATa mating strain Y4741 orfΔ::KanMAX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 and MATα mating strain, Y7092 can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu21Δ0 ura3Δ0 met15Δ0 were used. Yeast non-essential gene knockout collections [154], yeast over-expression plasmid library [155] and the collection of yeast gene-GFP fusion strains were utilized as before [29,31,33]. Yeast gene knockout was performed by PCR transformation using the Lithium Acetate method [156].

p281 construct carries a *LacZ* expression cassette under the control of a gal promoter. p281-4 construct carries an insert with a strong hairpin structure (5'GATCCTAGGATCCTAGGATCCTAGG ATCCTAG3') upstream of *LacZ* cassette[157]. pAG25 plasmid was used as a DNA template for nourseothricin sulfate (NAT) resistance gene marker in PCR reactions for gene knockout experiments. Kanamycin and NAT markers

were used as selection markers for corresponding deletion mutant strains. All plasmids carried an ampicillin resistance gene which was used as a selection marker in *E. coli* DH5α, and a *URA3* marker gene for selection in yeast.

P416 construct carries a *LacZ* expression cassette under the transcriptional control of a gal promoter. To generate reporter *LacZ* mRNAs under the translational control of complex RNA structures, three different fragments were cloned upstream of the *LacZ* mRNA in p416 construct using *XbaI* restriction site. In this way three expression constructs were designed as follows: pPGM2 construct contains the 5'-UTR of *PGM2* gene (5'TAATAAGAAAAAGATCACCAATCTTTCTCAGTAAAAAAAGAACAAAAGTTAACATAACAT 3'), pTAR construct contains the 5'-UTR of *HIV1-tar* gene (5'GGGTTCTCTGGTTAGCCAGATC TGAGCCCGGGAGCTCTCTGGCTAGCTAGGGAACCCACTGCTTAAGCCTCAATAAAGCTTGCCTG AGTGCTTCAAGTAGTGTGTGCC 3') and pRTN that contains the 5'-UTR of *FOAP-11* gene (5'GGGATTTTTACATCGTCTTGGTAAAGGCGTGTGACCCATAGGTTTTTTAGATCAAACACGTCT TTACAAAGGTGATCTAAGTATCTC 3').

YP (1% Yeast extract, 2% Peptone) or SC (Synthetic Complete) with selective amino acids (0.67% Yeast nitrogen base w/o amino acids, 0.2% Dropout mix,) either with 2% dextrose or 2% galactose, as a source of carbohydrates, was used as culture medium for yeast and LB (Lysogeny Broth) was used for *E. coli* cultures. 2% agar was used for all solid media. Yeast plasmid extraction was performed using yeast plasmid miniprep kit (Omega Biotek®) and *E. coli* plasmid extraction was carried out using GeneJET plasmid miniprep kit (Thermofisher® and Bio-Basics®) according to the manufacturers' instructions.

2.3.2 Drug sensitivity analysis

Yeast cells were grown from independent colonies to saturation for two days at 30°C in liquid YPgal. Serial dilutions of cell suspensions were spotted onto solid media with or without LiCl. Sensitivity to the compound was assessed by comparing the number and size of the colonies formed on each plate after 48 hours in comparison with wild type [30].

For quantification analysis, colony counting was done by taking 100 µL of diluted (10^{-4}) cell cultures from independent colonies, grown for 2 days at 30°C in liquid YPgal, and spreading on YPgal plates in the absence and presence of LiCl. The colonies were counted two days after incubation at 30°C. Each experiment was repeated at least three times. t-test analysis (*P*-value ≤ 0.05) was used to determine statistically significant results.

2.3.3 Quantitative *β-galactosidase* Assay

To evaluate the activity of *LacZ* expression cassette in different mutant strains, the quantitative *β-galactosidase* assay was performed using ONPG (O-nitrophenyl-α-D-galactopyranoside) as described [32,158]. Each experiment was repeated at least three times.

2.3.4 Quantitative real time PCR (qRT-PCR)

Deletion mutants in *PGM2*-GFP strain background were grown in YPgal overnight with or without LiCl treatment. Total RNA was extracted with Qiagen® RNeasy Mini Kit. Complementary DNA (cDNA) was made using iScript Select cDNA Synthesis Kit (Bio-Rad®) according to the manufacturer's instructions. cDNA was then used as a template for

quantitative PCR. qPCR was carried out using Bio-Rad® iQ SYBR Green Supermix and the CFX connect real time system (Bio-Rad®), according to the manufacturer's instructions. *PGK1* was used as a constitutive housekeeping gene (internal control).

The procedure was done in three repeats and t-test analysis (*P*-value ≤ 0.05) was used to determine statistically significant results. The following primers were used to quantify *PGM2* and *PGK1* mRNAs, as our positive control in different mutant strains.

PGM2: Forward GGTGACTCCGTCGCAATTAT; Reverse: CGTCGAACAAAGCACAGAAA

PGK1: Forward ATGTCTTTATCTTCAAAGTT; Revers: TTATTTCTTTTCGGATAAGA

2.3.5 Western blot analysis

Strains were grown in media treated with and without LiCl. Protein extraction was performed as described by Szymanski [159]. Bicinchoninic acid assay (BCA) was performed to estimate protein concentration as described by the manufacturer (Thermo Fisher®). Equal amounts of total protein extract (50 µg) were loaded onto a 10% SDS-PAGE gel, run on Mini-PROTEAN Tetra cell electrophoresis apparatus system (Bio-Rad®). Proteins were transferred to a nitrocellulose 0.45 µm membrane via a Trans-Blot Semi-Dry Transfer (Bio-Rad®). Mouse monoclonal anti-GFP antibody (Santa Cruz®) was used to detect protein levels of Pgm2p-GFP. Mouse anti-Pgk1 (Santa Cruz®) was used to detect Pgk1 protein levels used as internal controls. Immunoblots were visualized with chemiluminescent substrates (Bio-Rad®) on a Vilber Lourmat gel doc Fusion FX5-XT (Vilber®). Densitometry analysis was carried out using the FUSION FX software (Vilber®). Experiments were repeated at least three times; t-test analysis (*P*-value ≤ 0.05) was used to determine statistically significant results.

2.3.6 Genetic interaction analysis

Synthetic genetic analysis was performed in a 384 format as before [31,33,51]. In brief, deletion mutant for query gene in Mat α mating type was crossed to two sets of gene deletion mutants in Mat a mating type. After a few rounds of selection, double gene deletion mutants were selected in Mat a mating type. Colony size measurement was used to compare the fitness of double gene deletion mutants to single deletion strains. Colony size was measured as described before [24,54]. The colony size assay was repeated three times. For Phenotypic Suppression Array (PSA) analysis in SDL, a MATα yeast strain having an over-expression plasmid of our query gene is mated separately with each strain of the entire deletion set. with the library strains containing an empty plasmid was used as our reference for fitness control to address false positives. For phenotypic suppression analysis, the final constructs transformed into deletion library were grown on YPgal compared to the control plasmid. Phenotypic suppression array was performed by growing the transformed cells on YPgal with the sub-inhibitory concentration of LiCl (3 mM) as a stress condition drug [34]. We investigated the ability of over-expression of our query genes to compensate for the sick phenotype of our deletion sets under the inhibitory concentration of LiCl. If the over-expression of our candidate genes overcome the sensitivity of a yeast deletion strain caused by drug inhibition, we can suggest that a functional connection occurs between these two genes [30,160].

2.3.7 Genetic interaction Data Analysis

Scoring fitness was done by colony size measurement [24,54]. Those deletions that had 30% or more reduction in fitness in at least two experiments were considered hits. Based on their biological process and/or molecular function, hits were clustered into groups with enriched GO terms using Gene Ontology Resource http://geneontology.org/ and Genemania database http://genemania.org.

2.4 Results and Discussion

2.4.1 Deletion of *YTA6* and *YPR096C* increases yeast sensitivity to Lithium

Although the biology of BD has been extensively studied for decades, the molecular basis and the pathophysiology of this disorder is not clearly understood. It is known that DB causes significant neuroanatomical changes affecting frontal subcortical and prefrontal limbic brain regions [141]. However, how these changes occur in a mechanistic and at the molecular level has baffled scientists for years. LiCl has been used as an effective treatment option for BD for decades. Investigating the LiCl mode of action and its side effects as well as the molecular responses of a cell to LiCl may lead to a better understanding of BD's biology.

In mammalian systems, LiCl inhibits glycogen synthase kinase 3β. It also affects neurotrophic factors, neurotransmitters, and oxidative metabolism [161]. Numerous studies have also hypothesized alterations in gene expression as a result of LiCl [153]. In

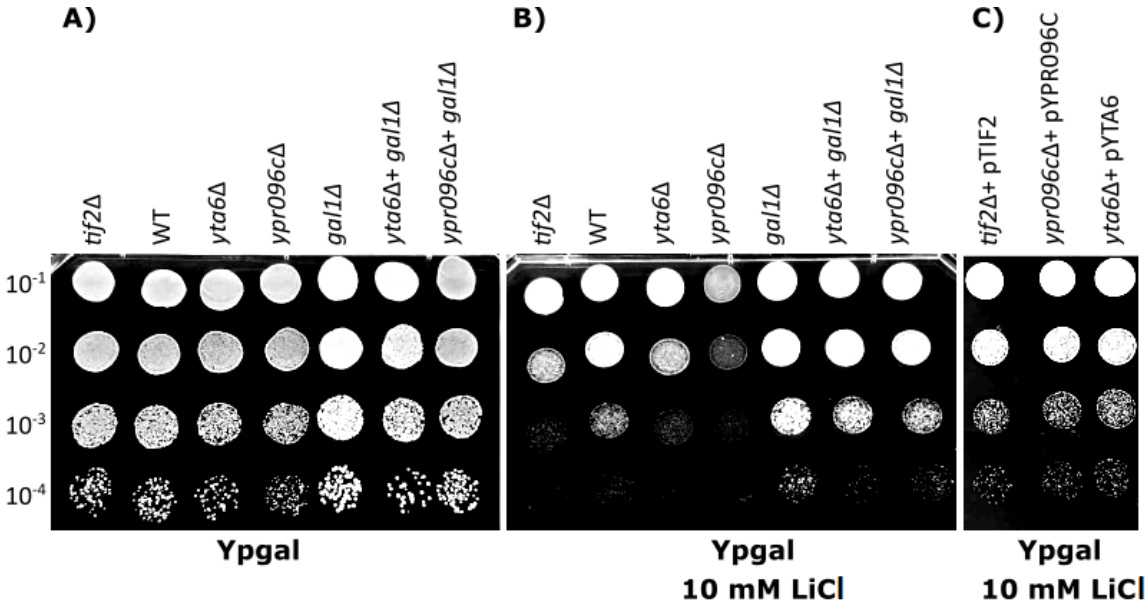
yeast *PGM2*, a key galactose metabolism gene is targeted by LiCl and over-expression of Translation Initiation Factor *TIF2* that codes for eIF4A, increases cell resistance to LiCl [148].

Drug sensitivity of mutant strains to a target chemical is an important tool to investigate how a chemical compound affects the cell at the molecular level and pathways influenced by the drug [31,33,57,162]. While investigating yeast gene deletion mutants that are sensitive to LiCl we identified two gene deletion mutants for *YTA6* and *YPR096C* that showed increased sensitivity to LiCl. Little is known about the molecular activity of these two genes and the cellular process in which they participate making them interesting targets to study. *YTA6* codes for a putative ATPase and *YPR096C* is an uncharacterized ORF.

In the spot test assay indicated in Fig 2.1 yta6Δ and ypr096cΔ show growth reduction in the presence of LiCl (10 mM LiCl) suggesting increased sensitivity of yeast strains when either of these two genes is deleted. *tif2Δ* was used as a positive control. To confirm that the observed phenotypic deficiency (sensitivity to LiCl) was due to the deletion of the target genes (*YTA6* and *YPR096C*) and not an effect of an unintended mutation in a secondary site, we re-introduced the ORFs back into their corresponding mutant strains. We observed that the re-introduction of the target genes recovered the fitness of the cells (Fig 2.1). To confirm the results obtained by the spot test assay we perform colony count measurement analysis, which represents a more quantitative approach. In this method, the decreased percentage of colonies is calculated by dividing the number of colonies in media in the presence of the LiCl to the number of colonies in control media and normalized to Wild Type (WT). Indicated in Fig 2 deletion of *YTA6*, *YPR096C* or *TIF2*

show reduced colony formation in the presence of LiCl. Reintroduction of the deleted genes into their corresponding gene deletion strains suppressed cell sensitivities to LiCl caused by gene deletions.

LiCl reduces the activity of phosphoglucomutase enzyme leading to the accumulation of galactose-1-phosphate, a toxic intermediate in galactose metabolism. To investigate the influence of *YTA6* and *YPR096C* on LiCl toxicity through galactose metabolism, we generated double gene deletions for *YTA6* or *YPR096C* with the *GAL1* gene. The deletion of *GAL1* gene relieved the sensitivity of gene deletion mutants for *YTA6* or *YPR096C* to LiCl (Figure 2.1). Also, the sensitivity of deletions strains for *YTA6* or *YPR096C* to LiCl diminished when glucose was used as a carbon source further connecting the observed LiCl sensitivity for *YTA6* and *YPR096C* deletion strains to galactose metabolism.



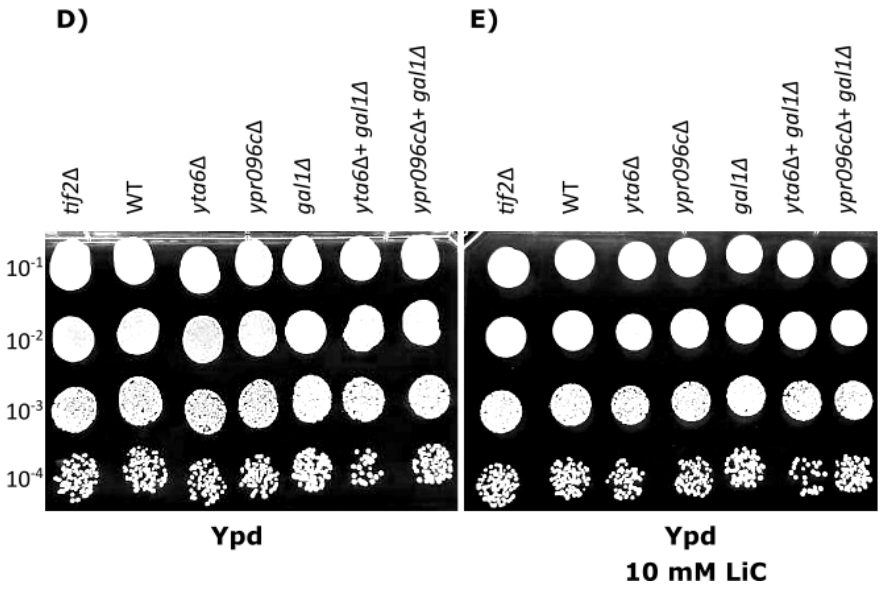


Figure 2.1. Drug sensitivity analysis for different yeast strains using spot test analysis. In (A) and (B) yeast cells were serially diluted as indicated (10^{-1} to 10^{-4}) and spotted on YPgal media with or without LiCl (10 mM). *yta6Δ* and *ypr096cΔ* show less growth under LiCl treatment. Double deletion for *GAL1* with *YTA6* or *YPR096C* suppressed the observed sensitivity of single-gene deletions for *YTA6* or *YPR096C.* The deletion of *TIF2* was used as a positive control. In (C) over-expression of the target gene in their corresponding deletion mutants reverted cell sensitivity to LiCl (10 mM). In (D) and (E) no LiCl toxicity was observed in media containing glucose as another carbon source.

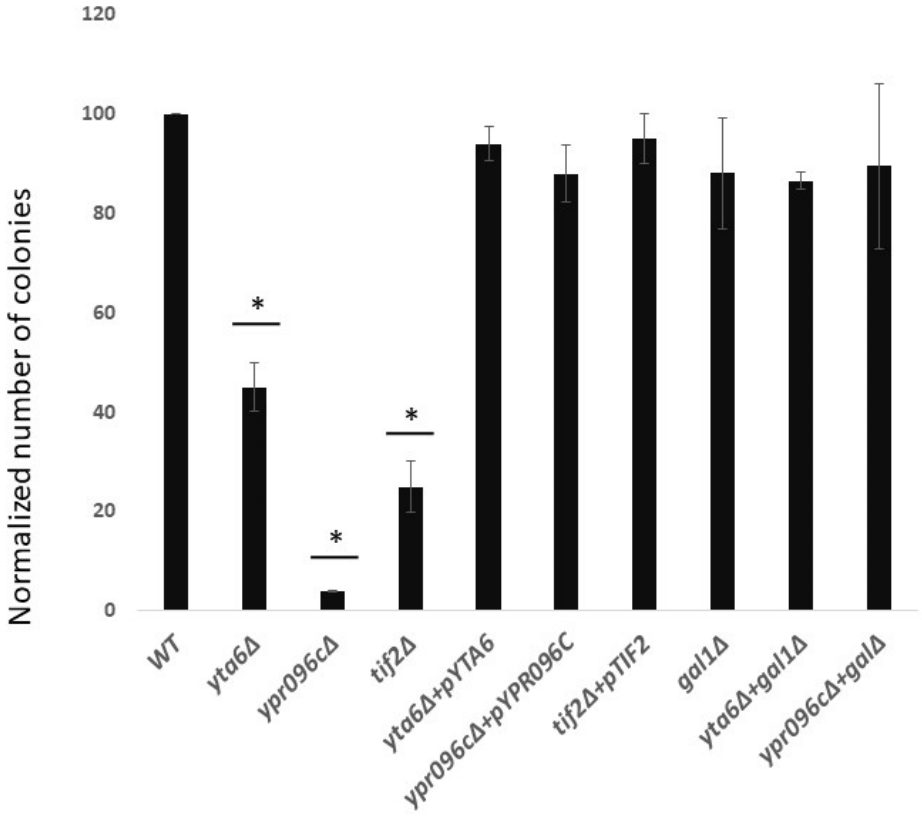


Figure 2.2. Quantitative analysis of drug sensitivity for different yeast strains. The average number of colonies formed for different yeast strains in the presence of LiCl (10 mM) was normalized to that for WT strain. Double deletion for *GAL1* with *YTA6* or *YPR096C* suppressed the observed sensitivity of single-gene deletions for *YTA6* or *YPR096C.* Data represent the average from three independent experiments and error bars represent standard deviation. * represent statistically significant results compared to the WT (*P*-value ≤ 0.05).

2.4.2 *YTA6* and *YPR096C* regulate the expression of *PGM2* at the level of translation.

PGM2 has been identified as a target of LiCl in yeast cells and its expression has been reported to change in the presence of LiCl [149]. Next, we investigated the ability of *YTA6* and *YPR096C* to change *PGM2* expression both at the levels of protein abundance (Figure

2.3A) and transcription (Figure 2.3B). This was done using western blot analysis in a strain where Pgm2p was tagged with a *GFP* gene. In the absence of LiCl, we observed no notable alteration in the Pgm2p levels when either *YTA6* or *YPR096C* were deleted. However, when cells were challenged with 10 mM LiCl, the deletion of either *YTA6* or *YPR096C* reduced the protein content of Pgm2p.

To investigate the possible effect of *YTA6* and *YPR096C* on *PGM2* transcription, we used qRT-PCR analysis to measure the content of *PGM2* mRNA when *YTA6* and *YPR096C* were deleted. Indicated in Figure 2.3B, the deletion of *YTA6* and *YPR096C* did not noticeably change the content of *PGM2* mRNA when cells were treated with LiCl. This suggests that *YTA6* and *YPR096C* are unlikely to alter *PGM2* expression at the transcription level. Together these observations connect the activities of *YTA6* and *YPR096C* to the expression of Pgm2p at the protein level. This is in agreement with a previous observation by Sofola-Adesakin et al. that in *Drosophila melanogaster* LiCl impaired gene expression at the protein synthesis level and not the mRNA level [145].

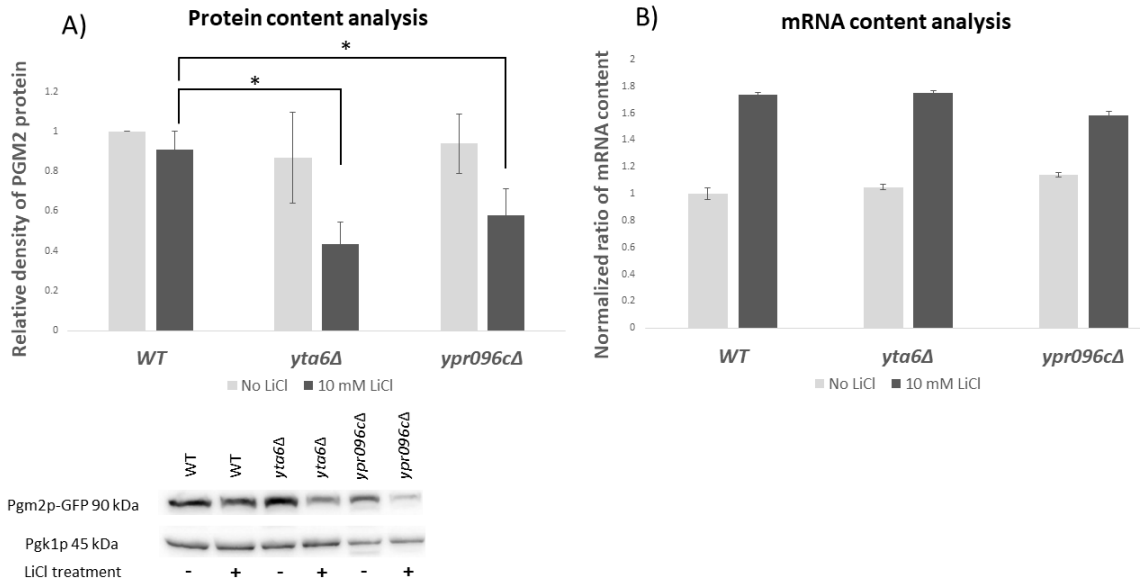


Figure 2.3. **Protein and mRNA content analysis** (A) Protein content analysis of Pgm2p-GFP protein in deletion of yeast strains for *yta6Δ* and *ypr096cΔ*. Western blot analysis was used to measure the protein content for Pgm2p-GFP protein in the absence or presence of LiCl (10 mM) and related to WT. Pgk1p was used as a housekeeping gene and the values are normalized to that. The inset represents a typical blot (B) mRNA content analysis of *PGM2* in *yta6Δ* and *ypr096cΔ*. qRT-PCR was used to evaluate the content of *PGM2* mRNA in yeast gene deletion mutants related to WT strain and normalized to *PGK1* mRNA levels in the absence or presence of LiCl (10 mM). Each experiment was repeated at least three times (n ≥ 3). Error bars represent standard deviation. * represent statistically significant results compared to the value in the corresponding WT. t-test analysis (*P*-value ≤ 0.05) was used to compare differences.

2.4.3 Translation of *β-galactosidase* reporter mRNA with a hairpin structure is altered by *YTA6* and *YPR096C*

The 5'-UTR of *PGM2* mRNA is predicted to contain a highly structured region [163] (Appendix Figure 8.1). This knowledge along with the observation that *YTA6* and *YPR096C* appear to impact *PGM2* expression at the protein level prompted us to investigate the influence of *YTA6* and *YPR096C* on the translation of other structured mRNAs. First, we placed the 5'-

UTR of *PGM2* mRNA in front of a *LacZ* reporter gene in a p416 expression construct [116]. Indicated in Figure 2.4 (A and B), when *YTA6* and *YPR096C* were deleted the activity of *β-galactosidase* was reduced for the reporter gene that contained 5'-UTR of *PGM2* mRNA and not a control mRNA without the 5'-UTR of *PGM2*. The deletion of *TIF2* was used as a positive control. Next, we utilized an expression cassette, p281-4 with a strong hairpin structure in front of a *LacZ* reporter gene [157]. A second construct, p281 without the hairpin structure was used as a control. Illustrated in Figure 2.4 (C and D) it was observed that when *YTA6* and *YPR096C* were deleted the activity of *β-galactosidase* was reduced for the reporter gene that contained a hairpin structure. When the hairpin was absent, the activity of *β-galactosidase* was independent of *YTA6* and *YPR096C*. Together these data show that the deletion of *YTA6* and *YPR096C* seem to reduce the translation of structured reporter mRNAs.

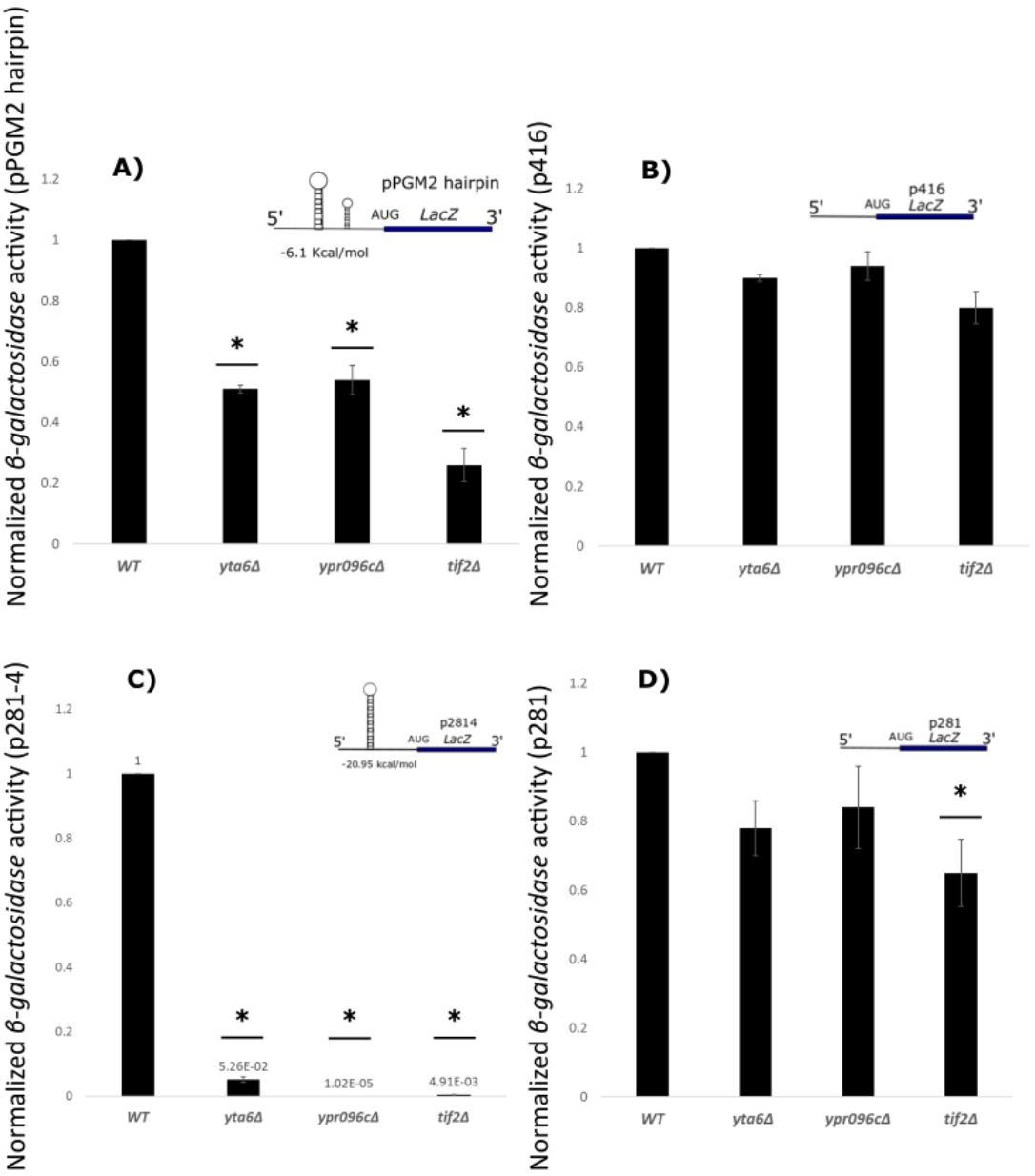


Figure 2.4. *β-galactosidase* expression analysis in different yeast strains. Activities from *β-galactosidase* mRNAs that carry 5'-UTR of *PGM2* mRNA (pPGM2) (A) or a strong hairpin structure (p281-4) (C) upstream of a *LacZ* reporter were reduced in *yta6Δ* and *ypr096cΔ* strains; *tif2Δ* was used as a positive control. Strains carrying low complexity regions upstream of *LacZ* reporters p416 (B) and p281 (D) did not show as significant reductions in *β-galactosidase* activity. Values are normalized to that for WT. Each experiment was repeated at least three times and error bars represent standard deviation. * represent statistically significant results (*P*-value ≤ 0.05). Insets represent schematic representation for the structure of the reporter mRNAs.

Next, we investigated the influence of *YTA6* and *YPR096C* on other structured mRNAs. For this, we designed two additional *β-galactosidase* mRNA reporters each carrying different complex RNA structures. pTAR carries the 5'-UTR of the *HIV1-tar* gene. This region contains a strong hairpin loop involved in modulating expression [102]. pRTN carries the 5'-UTR of the *FOAP-11* gene that contains a highly structured region [164]. Indicated in Figure 2.5, deletion strains for *YTA6* and *YPR096C* had a highly reduced level of *β-galactosidase* expression.

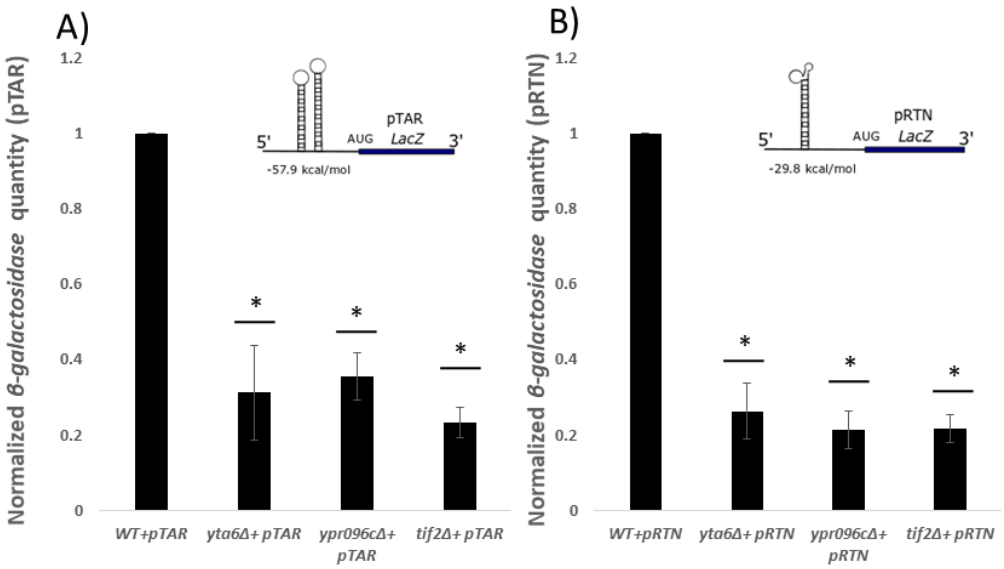


Figure 2.5. Normalized *β-galactosidase* activity is lower in *yta6Δ* and *ypr096cΔ* for structured mRNAs. pTAR (A) and pRTN (B) constructs contain the highly structured 5'-UTR of *HIV1-tar* and *FOAP-11* genes, respectively, in front of the *β-galactosidase* reporter mRNA. Values are normalized to that for WT. Each experiment was repeated at least three times and error bars represent standard deviation. * represent statistically significant results (*P*-value ≤ 0.05).

2.4.4 Genetic interaction analysis further connects the activity of *YTA6* and *YPR096C* to protein biosynthesis

Genetic interaction (GI) analysis is based on the assumption that parallel compensating cellular pathways give the cell its plasticity and tolerance against random deleterious mutations [24]. In this way, deletion of individual genes that can functionally compensate for each other has little or no phenotypic consequences. However, when both genes are deleted, an unexpected phenotype can emerge which can often be detected by a decrease in cell fitness or even cell death. In this case, the two genes are said to be forming a negative genetic interaction (nGI). An nGI can reveal the involvement of genes in compensating parallel pathways. nGI analysis has been used in various investigations to study gene function [29,31,34]. Systematic analysis of GIs in yeast is made possible by its two mating types. A target gene deletion in α-mating type (MAT alpha) is crossed with an array of single-gene deletion in a-mating type (MAT a) background and after a few rounds of selection double gene knockouts are obtained [51]. Colony size measurement is often used to determine the fitness of double gene knockouts [165]. To this end, we generated a set of double gene deletions mutants for our two query genes with 768 deletion mutants for genes involved in gene expression. This array was termed gene expression array. Due to inherent bias associated with such enriched subsets, a second set of double gene deletions were made for our query genes with 384 random gene deletions, termed random array, and was used as a control.

YTA6 formed 7 nGIs with different genes. The list of interactors includes *YPL079W* that encodes for large ribosomal subunit protein 21B and *YPL090C* that codes for small

ribosomal subunit 6A. *YPR096C* interacted with 8 genes including *YOR091W* that codes for a protein associated with translating ribosomes and *YOR078W* that codes for a protein involved in small ribosomal subunit biogenesis. The low number of nGIs observed for both *YTA6* and *YPR096C* makes it difficult to draw a statistically meaningful enrichment for the interacting genes. As a result, formulating function(s) for *YTA6* and *YPR096C* on the basis of the observed interactions is not feasible.

In addition, we investigated the conditional nGIs for the two target genes. Conditional GIs represent an interesting form of gene association. They represent a further insight into the function of genes under a specific condition. The activities of many genes are known to be condition dependent. For example, the expression of many DNA repair genes are regulated in response to DNA damage [28,166]. To this end, we investigated conditional nGIs for *YTA6* and *YPR096C* in the presence of a mild concentration of LiCl (3 mM). Illustrated in Figure 2.6 *YTA6* formed a total of 14 conditional nGIs. On the basis of their functions and cellular processes in which they participate, these genes can be divided into different categories. Of note, the category of genes involved in protein biosynthesis was the only significantly enriched category (P =1.6e-4). Within this category, we find 7 genes including, *RPL2B* that encodes large ribosomal subunit protein 2B and *YDR159W* that codes for a protein required for biogenesis of small ribosomal subunit. *YPR096C* formed 13 conditional nGIs, 6 of which belonged to the category of protein biosynthesis (P =6.6e-4). The genes in this category include *YDL081C* that codes for ribosomal stalk protein P1 alpha and *YER153C* that codes for a mitochondrial translation activator. The conditional nGIs observed here suggest a functional role for *YTA6* and *YPR096C* in protein

biosynthesis when cells are challenged with LiCl.

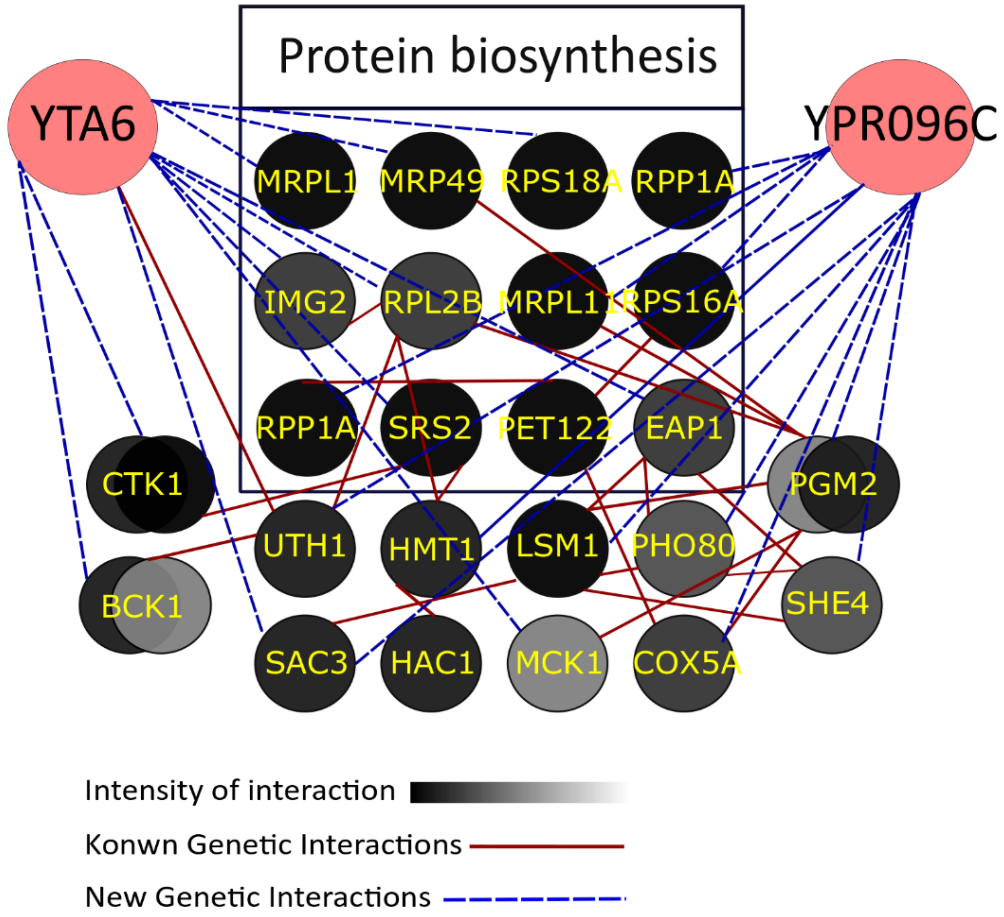


Figure 2.6. Conditional nGIs for *YTA6* and *YPR096C* under 3 mM concentration of LiCl. Our data shows a cluster of interactors involved in the protein biosynthesis pathway for *YTA6* (P =1.6e-4) and *YPR096C* (P =6.6e-4). *CTK1*, *HAC1*, *BCK1*, *MRPL1,* and *PGM2* are mutual hits shared between *YTA6* and *YPR096C.* Circles represent genes, dashed lines represent nGIs identified in this study and solid lines represent previously reported interactions in the literature.

PSA analysis focuses on another form of GIs, where a specific phenotype associated with a gene deletion mutant is suppressed by the over-expression of the second gene [7,167,168]. This type of GI generally indicates a close functional association where the activity of an

over-expressed gene compensates for the absence of the others. To this end, we subjected the gene expression array (described above) to 10 mM of LiCl. In this concentration, a number of strains showed sensitivity. We then attempted to reverse the observed sensitivities by over-expression of either *YTA6* or *YPR096C* in these mutants. Interestingly over-expression of either *YTA6* or *YPR096C* compensated for the sensitivity of the same two gene deletions, *bck1Δ* and *eap1Δ*, to LiCl (Figure 2.7). We confirmed our PSA data using spot test drug sensitivity analysis (Figure 2.7). We observed that sensitivity of *bck1Δ* and *eap1Δ* to 10 mM LiCl was relieved by introducing *pYTA6* and *pYPR096C* over-expression plasmids into deletion mutant strains (Figure 2.7). The fact that *YTA6* and *YPR096C* compensated the same two gene deletions, further connects their activities together in the context of LiCl sensitivity. *Bck1* is reported to function in cell wall integrity pathway and deadenylation of mRNAs and *Eap1* is an eIF4E-associated protein and accelerates the decapping of mRNAs. They have both been implicated in the regulation of alternative translation initiation via *Dhh1p*, a helicase protein [169–172]. *Dhh1p* is a member of the DEAD-box family of RNA helicases capable of unwinding strong secondary structures. It functions in mRNA decapping and translational repression among other processes [7,173]. A proposed functional association for both *YTA6* and *YPR096C* to the regulation of translation via *Dhh1* merits further investigations.

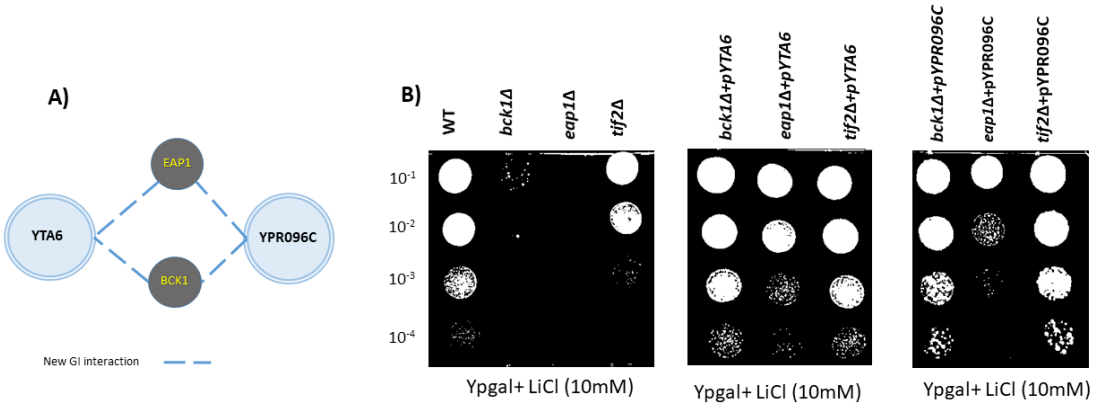


Figure 2.7. Over-expression of *YTA6* and *YPR096C* compensate for the sensitivity of *eap1Δ* and *bck1Δ* to 10 mM LiCl. (A) *BCK1* and *EAP1* are known to be involved in translation initiation via *DHH1* through previously reported genetic. New genetic interactions (PSA-based) identified in this study are shown with dashed lines. (B) Spot test analysis confirms the relief of drug sensitivity to LiCl for *eap1Δ and bck1Δ* by over-expression of *YTA6* and *YPR096C*.

3. Lithium chloride toxicity is connected to the regulation of gene expression in yeast.

3.1 Abstract

For decades, lithium chloride (LiCl) has been used as a treatment option for those living with bipolar disorder (BD). As a result, many studies have been conducted to examine its mode of action, toxicity, and downstream cellular responses. We know that LiCl is able to affect cell signaling and signaling transduction pathways through Protein Kinase C and Glycogen Synthase Kinase-3 which are considered to be important in regulating gene expression at the translational level. However, additional downstream effects require further investigation, especially in the translation pathway. In yeast, LiCl treatment affects the expression, and thus the activity, of *PGM2,* a phosphoglucomutase involved in sugar metabolism. However, it is not fully understood how LiCl affects gene expression in this matter. In this study, we identified three understudied genes, *NAM7*, *PUS2,* and *RPL27B* which increase LiCl sensitivity when deleted. We further demonstrate that *NAM7*, *PUS2,* and *RPL27B* are involved with the expression of *PGM2.*

3.2 Introduction

BD is known to be associated with dysregulated signaling pathways. LiCl has been a key treatment option for those living with BD demonstrating neuroprotective effects for more than six decades [174,175]. It has been used to prevent both depressive and manic episodes and is one of the only compounds available to have anti-suicidal effects in BD patients [143].

It has both pro- and anti-inflammatory effects, controlling the increase of inflammatory cytokines in psychiatric disorders [176].

In simplest terms, LiCl affects both the Protein Kinase C (PKC) and Glycogen Synthase Kinase-3 (GSK-3) signal transduction pathways, which are involved in translation, neurodevelopment, and neuronal plasticity [174]. It is proposed to induce proliferation of neural stem cells through Wnt signaling pathway [177]. Other pathways including cell proliferation, apoptosis, cell fate, and translation machinery are also known to be affected by LiCl [141,145,177,178].

Since its range of activities is so diverse, LiCl has been studied as a potential treatment for Alzheimer's disease [145,147]. Alzheimer's disease is well-known for its characteristic features of increased cell death in the dopaminergic regions of the brain due to increased plaques and tangles. In a study conducted by Yan [174] it was found that the inhibition of GSK-3β through LiCl treatments also had positive implications for adolescents facing the impact of methamphetamine exposure with the potential to prevent long-term behavioral alterations [174]. The efficacy of LiCl as a treatment for Alzheimer's disease appears to be linked to LiCl's ability to decrease levels of amyloid β and decrease phosphorylation of the tau protein. Both of these effects directly target symptoms observed in patients with Alzheimer's disease [179]. LiCl has also been investigated as a treatment option for Parkinson's disease and Huntington's disease [145,147].

Although much has been learned about the influence of LiCl, it is still unclear how it affects the cell at the molecular level. The effects of LiCl on gene expression and its secondary

effects are also not well-known. It's been observed that after long term treatment of BD patients with LiCl, it affects gene expression of genes involved in the PI/PKC signaling cascade, consequently causing disruption in the function of nerve cells [140,144–146], renal cells and liver cell damages [175] which requires further investigation of the mechanism(s) of its activity, as well as secondary effects [140,141,147].

Yeast is particularly sensitive to LiCl treatment because it alters the expression and activity of *PGM2*, a phosphoglucomutase that mediates the entry of galactose into glycolysis [148,150]. *PGM2* also converts glucose-1-P to glucose-6-P, and if inhibited, it leads to the accumulation of glucose-1-P causing toxicity in yeast cells [151]. When LiCl is added to galactose media, yeast cell growth is severely reduced due to impaired glycolysis. LiCl also reduces the levels of UDP-glucose and disrupts associated pathways in the presence of glucose. Other studies have also suggested that LiCl might inhibit RNA processing enzymes and rapidly reduce the expression of ribosomal protein genes (RBG) [152,153]. This results in a decreased number of mature mRNAs in the cytoplasm [153] suggesting inhibition at the translational level.

Studies are also being done to see if LiCl inhibits the initial steps of protein synthesis. One study has demonstrated that LiCl may be able to disrupt the association of translation initiation factor eIF4A, an RNA helicase, to the rest of the translational machinery, impairing translation initiation [148]. However, this was not observed in yeasts growing on glucose media as a carbon source.

NAM7 is an important gene involved in efficient termination of translation, especially in nonsense-mediated mRNA decay. This gene codes for an RNA helicase that binds to the small ribosomal subunit. *PUS2* is involved in tRNA modification in the mitochondria and also pseudouridylation of some nuclear mRNAs. *And RPL27B* encodes for the large ribosomal subunit. In follow-up genetic studies, we suggest that the deletion of *NAM7, PUS2*, and *RPL27B* increases yeast sensitivity to LiCl through the expression of *PGM2*. In this study, we demonstrate that the deletion of yeast genes *NAM7, PUS2*, and *RPL27B* increases the sensitivity of yeast to LiCl. These genes have never been studied to cell responses linked to LiCl before.

3.3 Materials and Methods

3.3.1 Strains, plasmids, gene collections and cell and DNA manipulations

MATa mating strain Y4741 orfΔ::KanMAX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 and MATα mating strain, Y7092 can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu21Δ0 ura3Δ0 met15Δ0 were used. Deleted mutant strains were obtained from yeast non-essential gene knockout mutant library [50]. This library was also used for Synthetic Genetic Analysis to make double mutants, overexpression plasmid of candidate genes were extracted from yeast over-expression collection [155] and yeast PGM2-GFP fusion strain was purchased from Yeast GFP Clone Collection from Thermofisher® which was used for qPCR and western analysis purposes as described before [29,31,33]. Yeast gene deletions in MATα strain were performed by PCR transformation and homologous recombination through the Lithium Acetate method as described in previous experiments [156].

p281 construct, carrying a *LacZ* expression cassette under the control of a gal promoter, was used as our control plasmid. p281-4 with a strong hairpin (5' GATCCTAGGATCCTAGG ATCCTAGG ATCCTAG 3'), inserted upstream of *LacZ* expression cassette was used as our investigating construct [157]. pAG25 plasmid was used as a DNA template in PCR reactions to amplify nourseothricin sulfate (NAT) resistance gene marker, to identify successful transformation in gene knockout experiments. All plasmids carried an ampicillin resistance gene which was used as a selection marker in *E. coli* DH5α, and a URA3 marker gene for selection in yeast. Kanamycin and NAT drug was used as selectable markers for gene knock out in Mat "a" and Mat "α" respectively.

p416 construct carries a *LacZ* expression cassette under the transcriptional control of a gal promoter which was used as our plasmid control in translation activity assays. To generate reporter *LacZ* mRNAs under the translational control of complex RNA structures, three different fragments were cloned upstream of the *LacZ* mRNA in p416 construct using XbaI restriction site. Using this method, three expression constructs were designed as follows: pTAR construct which contains the 5'-UTR of HIV1-tar gene (5'GGGTTCTCTGGTTAGCCAGATCTGAGCCCGGGAGCTCTCTGGCTAGCTAGGGAACCCACTGC TTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCAAGTAGTGTGTGCC 3') and pRTN which contains the 5'-UTR of FOAP-11 gene (5'GGGATTTTTACATCGTCTTGGTAAAGGCGTGTGAC CCATAGGTTTTTTAGATCAAACACG TCTTTACA AAGGTGATCTAAGTATCTC 3').

YP (1% Yeast extract, 2% Peptone) or SC (Synthetic Complete) with selective amino acids (0.67% Yeast nitrogen base w/o amino acids, 0.2% Dropout mix) either with 2% dextrose or 2% galactose, as a carbon source, were used as culture medium for yeast and LB

(Lysogeny Broth) was used for *E. coli* cultures. 2% agar was used for all solid media. Yeast plasmid extraction was performed using yeast plasmid miniprep kit (Omega Biotek®) and *E. coli* plasmid extraction was carried out using GeneJET plasmid miniprep kit (Thermofisher® and Bio-Basics®) according to the manufacturers' instructions.

3.3.2 Drug sensitivity analyses

To over saturate yeast cells for spot test analysis, cultures were grown in liquid medium for two days at 30°C shaker incubator. Following incubation, four serial dilutions (10^{-1} to 10^{-4}) of the cell cultures were spotted onto solid media with or without LiCl treatment. After 48 hours, the sensitivity of was determined based on their colony size and number [30].

For drug sensitivity colony count assays, 100 µL of diluted (10^{-4}) oversaturated cell culture, as described above, was spread on YPgal plates in the absence and presence of LiCl. Plates were then analyzed based on colony numbers after incubation at 30°C incubator for two days. Each experiment was repeated at least three times. *t*-test analysis (p-value ≤ 0.05) was used to determine statistically significant results from WT control.

3.3.3 Quantitative *β-galactosidase* Assay

This assay was used to evaluate the activity of *LacZ* cassette in different mutant strains as a reference for translation activity. The quantitative *β-galactosidase* assay was performed using ONPG (O-nitrophenyl-α-D-galactopyranoside) as described by Krogan et al., [32] . Each

experiment was repeated at least three times.

3.3.4 Quantitative real time PCR (qRT-PCR)

To determine the mRNA level of PGM2 in different deletion mutants, *PGM2*-GFP yeast strain was grown in YPgal overnight with or without LiCl treatment. Total RNA was extracted with Qiagen® RNeasy Mini Kit. Complementary DNA (cDNA) was made using iScript Select cDNA Synthesis Kit (Bio-Rad®) according to the manufacturer's instructions. cDNA was then used as a template for quantitative PCR. qPCR was carried out using Bio-Rad® iQ SYBR Green Supermix and the CFX connect real time system (Bio-Rad®), according to the manufacturers' instructions. *PGK1* was used as a constitutive housekeeping gene (internal control).

The procedure was done in three repeats and t-test analysis (*P*-value ≤ 0.05) was used to determine statistically significant results. The following primers were used to quantify *PGM2* and *PGK1* mRNAs, as our positive control in different mutant strains.

PGM2: Forward GGTGACTCCGTCGCAATTAT; Reverse: CGTCGAACAAAGCACAGAAA

PGK1: Forward ATGTCTTTATCTTCAAAGTT; Revers: TTATTTCTTTTCGGATAAGA

3.3.5 Western blot analysis

Deleted mutant strains with GFP-tagged *PGM2* were grown in media treated with and without LiCl to investigate protein levels of PGM2 in different conditions. Protein extraction was performed as described by Szymanski et al., [159]. Bicinchoninic acid assay (BCA) was performed to estimate protein concentration as described by the manufacturer

(Thermo Fisher®). Equal amounts of total protein extract (50 µg) were loaded onto a 10% SDS-PAGE gel, run on Mini-PROTEAN Tetra cell electrophoresis apparatus system (Bio-Rad®). Proteins were transferred to a nitrocellulose 0.45 µm membrane via a Trans-Blot Semi-Dry Transfer (Bio-Rad®). Mouse monoclonal anti-GFP antibody (Santa Cruz®) was used to detect protein levels of Pgm2p-GFP. Mouse anti-Pgk1 (Santa Cruz®) was used to detect Pgk1p levels, which was used as an internal control. Immunoblots were visualized with chemiluminescent substrates (Bio-Rad®) on a Vilber Lourmat gel doc Fusion FX5-XT (Vilber®). Densitometry analysis was carried out using the FUSION FX software (Vilber®). Experiments were repeated at least three times. *t*-test analysis (p-value ≤ 0.05) was used to determine statistically significant results.

3.3.6 Genetic interaction analysis

Synthetic genetic analysis was performed in a 384 format as described previously [31,51,165] to investigate genetic interactions (GIs) of candidate genes. GI is presumed when double deletion phenotype is different from single deletion [53], suggesting the deleted genes to be functionally interacting in parallel pathways, causing sick or lethality. Essentially, the candidate gene is deleted in Mat "α" mating type and then crossed to three sets (≈ 1000) of single mutant Mat "a" mating type selected from the single mutant library of yeast. After a few rounds of selection, double gene deletion mutants were selected in Mat a mating type. Fitness or colony size of double mutant strains were compared to that of single mutant strains to detect synthetic sickness or synthetic lethal [24,54]. The experiment was repeated three times.

For Conditional SGA analysis, we grew our double mutant strain on media with a sub-inhibitory concentration of LiCl (3 mM) investigating genes that are expressed only under LiCl treatment [28,33].

For Phenotypic Suppression Array (PSA) analysis, we mated Mat "α" carrying over-expression plasmid of our query gene to three sets (≈ 1000) of single mutant Mat "a" selected from the single mutant library of yeast, similar to SGA procedure as mentioned above. In the last step, Mat "a" strains carrying over-expression plasmid are compared to that of Mat "a" carrying a control plasmid [34], both grown in media treated with LiCl. Possible GIs were selected based on sick or lethal phenotype when grown on media with LiCl treatment and were compensated with the introduction of over-expression plasmid. In this matter, we can propose a functional connection between these two genes [30,160].

3.3.7 Genetic interaction Data Analysis

Scoring fitness was done using colony size measurement as described previously [24,54]. We set our threshold at fitness ≤ 70% compared to control. Each experiment was repeated at least three times, and mutual hits observed at least two times were selected. A list of hits then categorized based on their biological process and/or molecular function, with enriched GO terms using Gene Ontology Resources: http://geneontology.org/, Genemania database http://genemania.org and profiling of complex functionality http://webclu.bio.wzw.tum.de/profcom/start.php.

3.4 Results

3.4.1 Deletion of *NAM7, PUS2* and *RPL27B* increases yeast sensitivity to Lithium Chloride.

As mentioned above, we know that in *PGM2* is an important enzyme in yeast galactose metabolism, which is targeted by LiCl. We also know that over-expression of Translation Initiation Factor *TIF2*, which codes for eIF4A, increases cell resistance to LiCl [148]. In the spot test assay, we showed that the deletion of *TIF2* causes increased sensitivity when grown in the presence of 10 mM LiCl. Deletion of *NAM7, PUS2* and *RPL27B* showed dramatic growth reduction in the same manner (Figure 3.1B). This suggests that the deletion of any of these three genes increases sensitivity to LiCl. It was previously shown that over-expression of *TIF2* revert LiCl toxicity [148], and to show if our candidate genes would revert LiCl sensitivity in the same manner, we transferred over-expression plasmid of our candidate genes into deletion strains of their mutants, spotted on 10 mM LiCl. When the plasmids were introduced to the mutants, the fitness of the strains was recovered, proposing they may have a similar function as eIF4A in the cell (Figure 3.1C). We showed that indeed, the deletion of *TIF2*, which codes for eIF4A, increased sensitivity to LiCl in yeast, and it was shown that over-expression of eIF4A was able to revert the inhibition of translation due to LiCl [148].

These spot test assay findings were confirmed with colony count measurement analysis, providing a quantitative analysis of our results (Figure 3.2). In this experiment, the number of colonies seen in the presence of LiCl in the media is compared to the number of colonies seen in control media. This is normalized to the wild type. We were able to

calculate the decreased percentage of colonies using this data. As seen in Figure 3.2, the deletion of *NAM7, PUS2, RPL27B,* or *TIF2* lead to decreased colony formation.

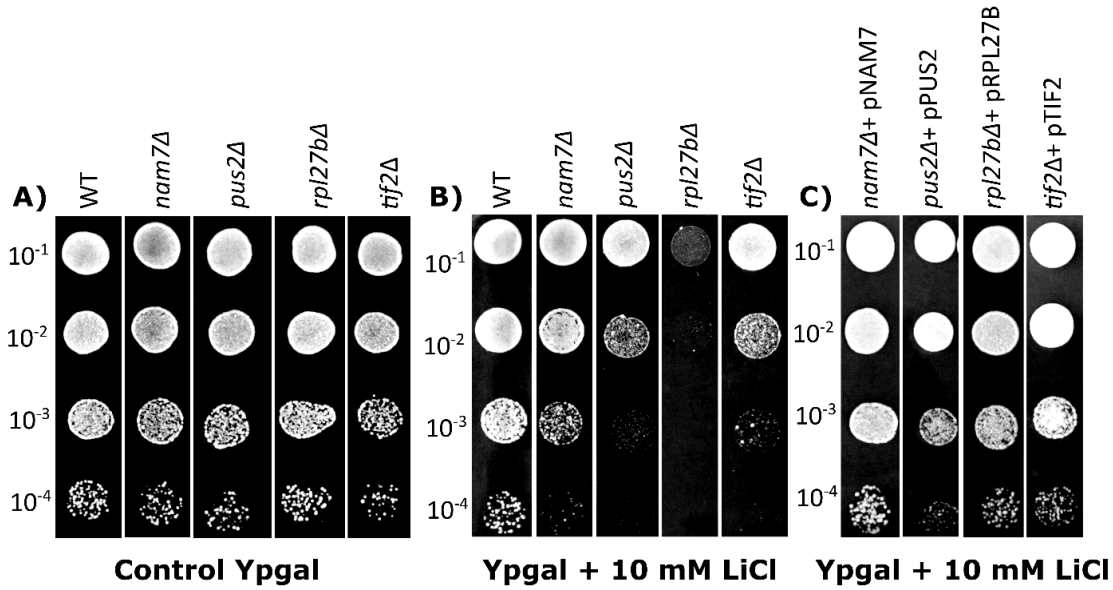


Figure 3.1. Drug sensitivity analysis for different yeast strains using spot test analysis. In (A), (B) and (C) yeast cells were serially diluted as indicated (10^{-1} to 10^{-4}) and spotted on YPgal media with or without LiCl (10 mM). in (A) and (B) *nam7Δ, pus2Δ* and *rpl27bΔ* show less growth under LiCl treatment. The deletion of *TIF2* was used as a positive control. (C) Over-expression of the target gene in their corresponding deletion mutants reverted cell sensitivity to LiCl (10 mM).

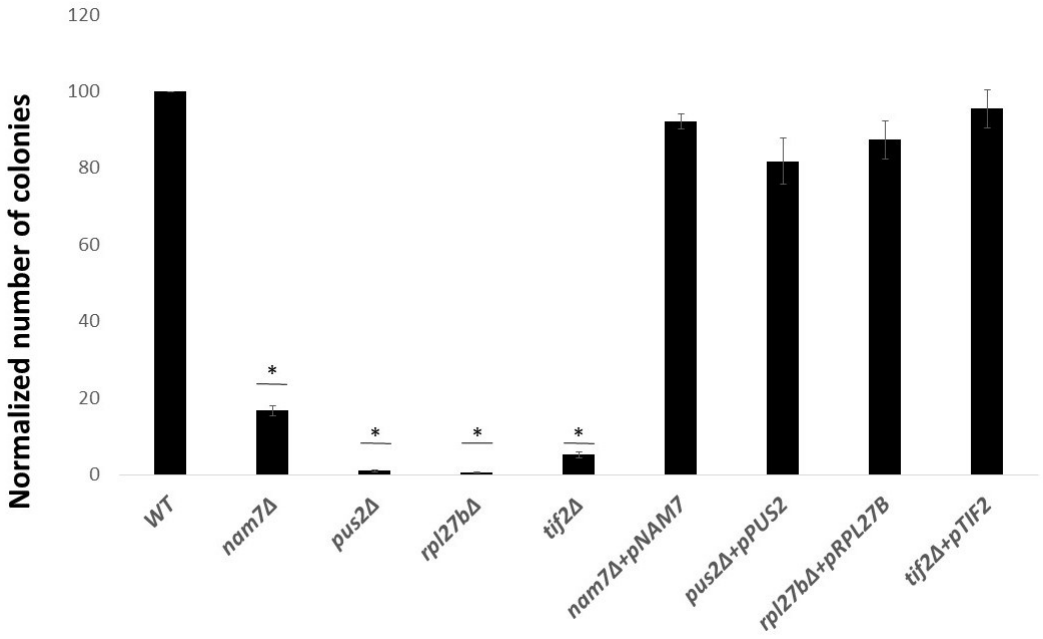


Figure 3.2. Quantitative analysis of drug sensitivity for different yeast strains. The average number of colonies formed for different yeast strains in the presence of LiCl (10 mM) was normalized to that for WT strain. *nam7Δ, pus2Δ,* and *rpl27bΔ* show fewer colonies under LiCl treatment. The deletion of *TIF2* was used as a positive control. Over-expression of target genes in their corresponding deletion mutants reverted cell sensitivity to LiCl. *(*P*-value ≤ 0.05) represent statistically significant results from WT control. Data represents the average from three independent experiments and error bars represent standard deviation.

3.4.2 *NAM7, PUS2,* and *RPL27B* regulate the expression of *PGM2* at the level of translation.

Previous studies have shown that LiCl exposure alters the expression of *PGM2*, a phosphoglucomutase crucial to galactose metabolism [148–150]. This enzyme plays two important roles: the facilitation of galactose into glycolysis and the conversion of glucose-1-phosphate to glucose-6-phosphate.

Since altered expression of *PGM2* has been seen in the presence of LiCl, we examined to see if *NAM7, PUS2,* or *RPL27B* would affect *PGM2* expression at the translation level.

Pgm2p was tagged with GFP and western blot analysis was done to see if any of the three genes altered *PGM2* expression (Figure 3.3A). When cells were exposed to 10 mM LiCl, the deletion of any three of the genes resulted in reduced protein levels of Pgm2p. When cells were not exposed to LiCl, the deletion of *NAM7, PUS2,* or *RPL27B* showed no observable difference.

To see if *NAM7, PUS2,* or *RPL27B* impact *PGM2* transcription levels, qRT-PCR was performed to measure the expression of *PGM2* mRNA. As seen in Figure 3.3B, the deletion of *NAM7, PUS2,* or *RPL27B* did not significantly alter *PGM2* mRNA levels when exposed to LiCl, suggesting that these genes do not impact *PGM2* at the transcriptional level. Thus, this would imply that *NAM7, PUS2,* or *RPL27B* affect the expression of Pgm2p at the protein level. These results are in agreement with a previous study by Sofola-Adesakin [145]which demonstrated that LiCl impaired gene expression during protein synthesis and not during transcription.

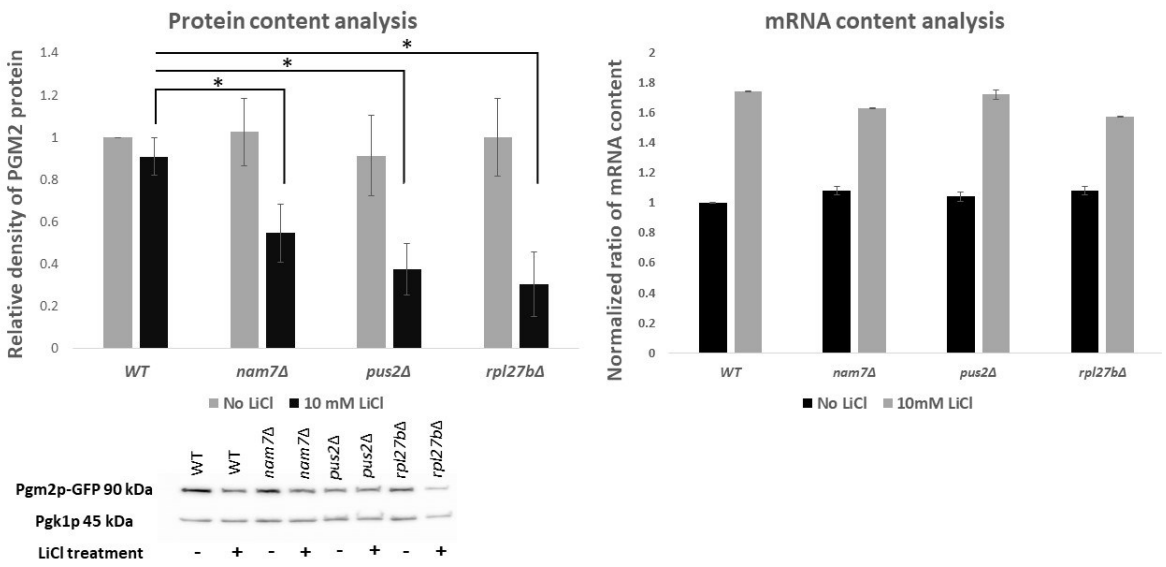


Figure 3.3. Protein and mRNA content analysis. (A) Protein content analysis of Pgm2p-GFP protein in deletion of yeast strains for *nam7Δ, pus2Δ,* and *rpl27bΔ*. Western blot analysis was used to measure the protein content for Pgm2p-GFP protein in the absence or presence of LiCl (10 mM). Pgk1p was used as a housekeeping gene and the values are normalized to that. (B) mRNA content analysis of *PGM2* in *nam7Δ, pus2Δ,* and *rpl27bΔ*. qRT-PCR was used to evaluate the content of *PGM2* mRNA in yeast gene deletion mutants related to WT strain and normalized to *PGK1* mRNA levels. mRNA level shows to increase in treatment of liCl but there is no significant difference between mutants and WT. Each experiment was repeated at least three times. * (*P*-value ≤ 0.05) represent statistically significant results compared to WT coontrol. Error bars represent standard deviation.

3.4.3 Translation of *β-galactosidase* reporter mRNA with a hairpin structure is altered by *NAM7, PUS2,* and *RPL27B.*

Previous studies have predicted that the 5' end of *PGM2* mRNA has a highly structured region [163] (Appendix Figure 8.1). *PGM2* expression is severely reduced in the absence of translation initiation helicase *TIF2*, a protein responsible for unwinding mRNA structures during translation [148]. Since we saw that *NAM7, PUS2,* and *RPL27B* are likely to work at

the translational level, we wanted to see if these genes are possibly impacting the translation of highly structured mRNAs. For this experiment, we inserted 5'-UTR of *PGM2* in form of *LacZ* cassette of p416 plasmid (pPGM2). Then pPGM2 was transformed into deletion of mutant strains of our candidate genes and WT and *β-galactosidase* activity was measured as a reference for translation activity (Fig 4.4A). It was shown that translation activity significantly decreased in *Nam7Δ*, *Pus2Δ,* and *Rpl27bΔ* carrying a plasmid with hairpin when compared to translation rate with the control plasmid (Figure 3.4B).

To support our findings, we used another construct with a strong hairpin structure at 5'-UTR (p281-4) [157] to show if query genes were most likely affecting highly structured 5'-UTRs and not only *PGM2*. Our control was p281 without the hairpin structure [157]. We observed that with the deletion of *NAM7, PUS2,* or *RPL27B, β-galactosidase* activity was reduced in p281-4, whereas p281 had no observable difference (Figure 3.4A and 3.4B). These results demonstrate that these genes are not affecting the general translation of mRNAs lacking highly structured mRNAs.

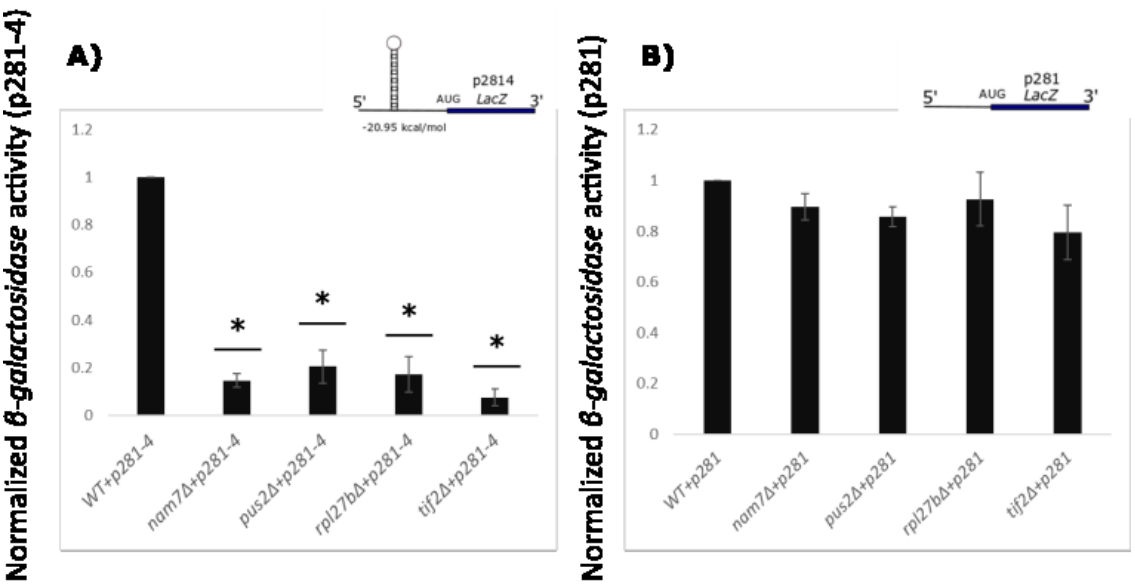


Figure 3.4. *β-galactosidase* expression analysis in different yeast strains. (A) Activities from *β-galactosidase* mRNAs that carry a strong hairpin structure (p281-4) upstream of a *LacZ* reporter were highly reduced in *nam7Δ, pus2Δ,* and *hal1Δ* strains; *tif2Δ* was used as a positive control. (B) Strains carrying low complexity (p281) upstream of the *LacZ* gene did not show reductions in *β-galactosidase* activity. Values are normalized to that for WT. Each experiment was repeated at least 3 times and error bars represent standard deviation. * (*P*-value ≤ 0.05) represent statistically significant results from WT control. Insets represent schematic representation for the structure of the reporter mRNAs.

Since *NAM7, PUS2,* and *RPL27B* impacted translation of structured reporter mRNAs, we investigated if they were able to impact the translation of other naturally structured mRNAs. To do this, we designed two *β-galactosidase* mRNA reporters with different complex RNA structures. pTAR has the 5'-UTR of the *HIV1-tar* gene, which has a strong hairpin loop, while pRTN has the 5'-UTR of the *FOAP-11* gene, which has a highly structured region [102,164]. When *NAM7, PUS2,* or *RPL27B* were deleted, levels of *β-galactosidase* expression were significantly reduced (Figure 3.5).

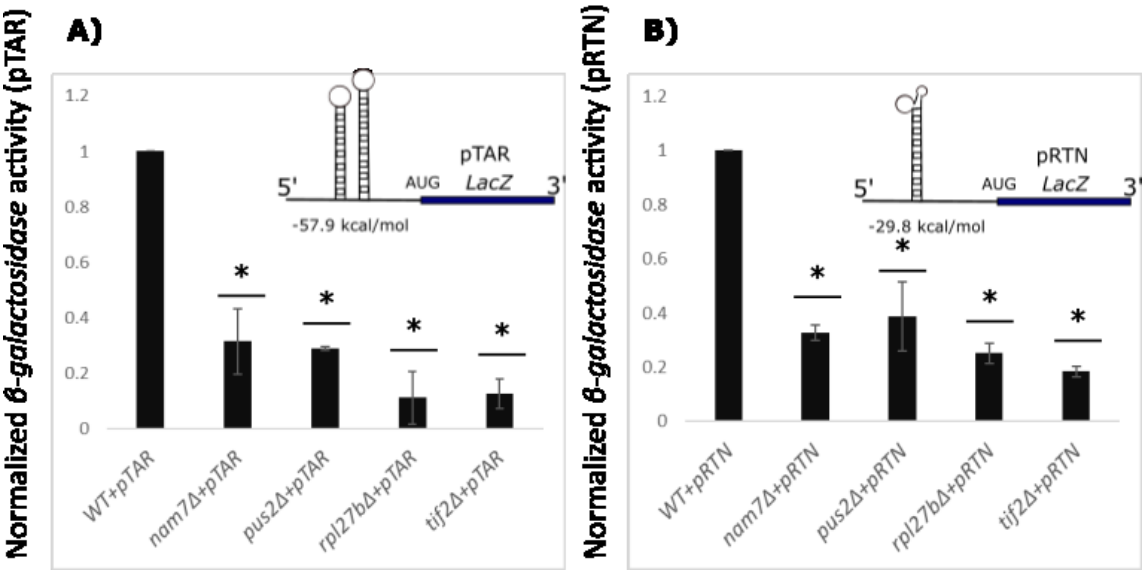


Figure 3.5. Normalized *β-galactosidase* activity is lower in *nam7Δ, pus2Δ* and *rpl27bΔ* for structured mRNAs. pTAR (A) and pRTN (B) constructs contain the highly structured 5'-UTR of *HIV1-tar* and *FOAP-11* genes, respectively, in front of the *β-galactosidase* reporter mRNA. Values are normalized to that for WT. *(*P*-value ≤ 0.05) represent statistically significant results from WT control. Each experiment was repeated at least 3 times and error bars represent standard deviation.

3.4.4 Genetic interaction analysis further connects the activity of *NAM7, PUS2,* and *RPL27B* to protein biosynthesis.

Genetic interaction (GI) analysis assumes that parallel pathways allow for plasticity and tolerance against random deleterious mutations, protecting cells from certain death if one gene is deleted or mutated in a pathway [24]. This means that a gene in one pathway can compensate for the lack of gene activity in a parallel pathway, allowing the cell to survive. Accordingly, when two genes in parallel pathways are deleted, cell fitness decreases or the cell dies. As a result, we can propose they are having genetic interaction, or in other words, they are functionally working in parallel pathways called negative genetic interactions (nGIs). nGIs are useful in many studies to understand gene function

[29,31,34].

Analysis of GIs in yeast is done by mating two types of yeast: α-mating type (Mat α), and a-mating type (Mat a). Mat α is the target gene deletion, which is crossed with Mat a, an array of single-gene deletions to produce double gene deletions [51]. The fitness of double deletions is measured using colony size assessment [165]. Using this method, we made double deletions for our three genes, observing the genetic interactions of *NAM7, PUS2,* and *RPL27B* with nearly 1000 other genes that may interact with our candidates. In this experiment, 384 genes were randomly selected form the mutant library as controls.

We observed 33 nGIs with *NAM7*, including *PRP22, TIF2, GCD11*, and *PRT1. PRP22* is a DEAH-box RNA helicase, *TIF2* codes for the translation initiation factor eIF4A, *GCD11* forms part of the small subunit of eIf2, and *PRT1* is the subunit of eIf3. Overall, 36% of the genes that had nGIs with *NAM7* are involved in translation, many of which are involved in translation initiation factor activity including *YGR054W* and *HCR1* (Figure 3.6).

PUS2 had 35 nGIs including *DHH1, EAP1*, and *HCR1. DHH1* codes for an ATP-dependent RNA helicase. *EAP1* codes for an eIF4E-associated protein, and *HCR1* codes for a subunit of the eIF3 translation initiation factor, which is also important in the binding of initiation factors to the 40S subunit and AUG recognition along with *HCR1* as an RNA recognition motif [180]. 31% of *PUS2* nGIs were involved in ribosomal structural units. *XRN1*, *YOR302W*, *VIP1,* and *WHI3,* along with the other mentioned genes, are involved in the regulation of translation (Figure 3.6).

RPL27B had 54 nGIs, 25% of which are involved in translation and 16% of which are involved in translation regulation (Figure 3.6). This included *DHH1, EAP1, SLH1* and *SKI2* with RNA helicase activity, and *ECM32* as a DNA helicase enzyme, modulating translation termination.

Comparing *NAM7*, *PUS2,* and *RPL27B* using a genetic interactor revealed some interesting mutual hits being important mainly in translation regulation. *HCR1* is a subunit of eIF3 and is known to be important for scanning efficiency especially in cooperation with *DED1* (RNA helicase) on long 5'-UTRs [131] and binding with *DHX29* in human cells [181]. *HCR1*, another mutual hit, and *PRT1* are crucial in recognition of the right start codon during scanning by inhibiting leaky scanning through promoting the stability of ribosomes on mRNA [182,183]. *XRN1* is known to be involved in mRNA decay and transcription regulation but recently was proposed that it might also play a role in the translation pathway by regulating translation of specific mRNAs through binding to eIF4F complex a translation IC [184]. *DED1* is an ATP-dependent helicase that associates with eIF4A to regulate translation initiation [94,134]. Methylation of *DED1* strengthens its binding to eIF4A and to *XRN1* [101], suggesting their potential effect on translation regulation. *XRN1* was also proposed to interact with another helicase *DHH1* in yeast to control the negative regulation of translation by decapping mRNAs for degradation [97,185]. *P54* (homolog of *DED1* in humans) was shown to be important in localization and assembly of P-bodies in the cell [97].

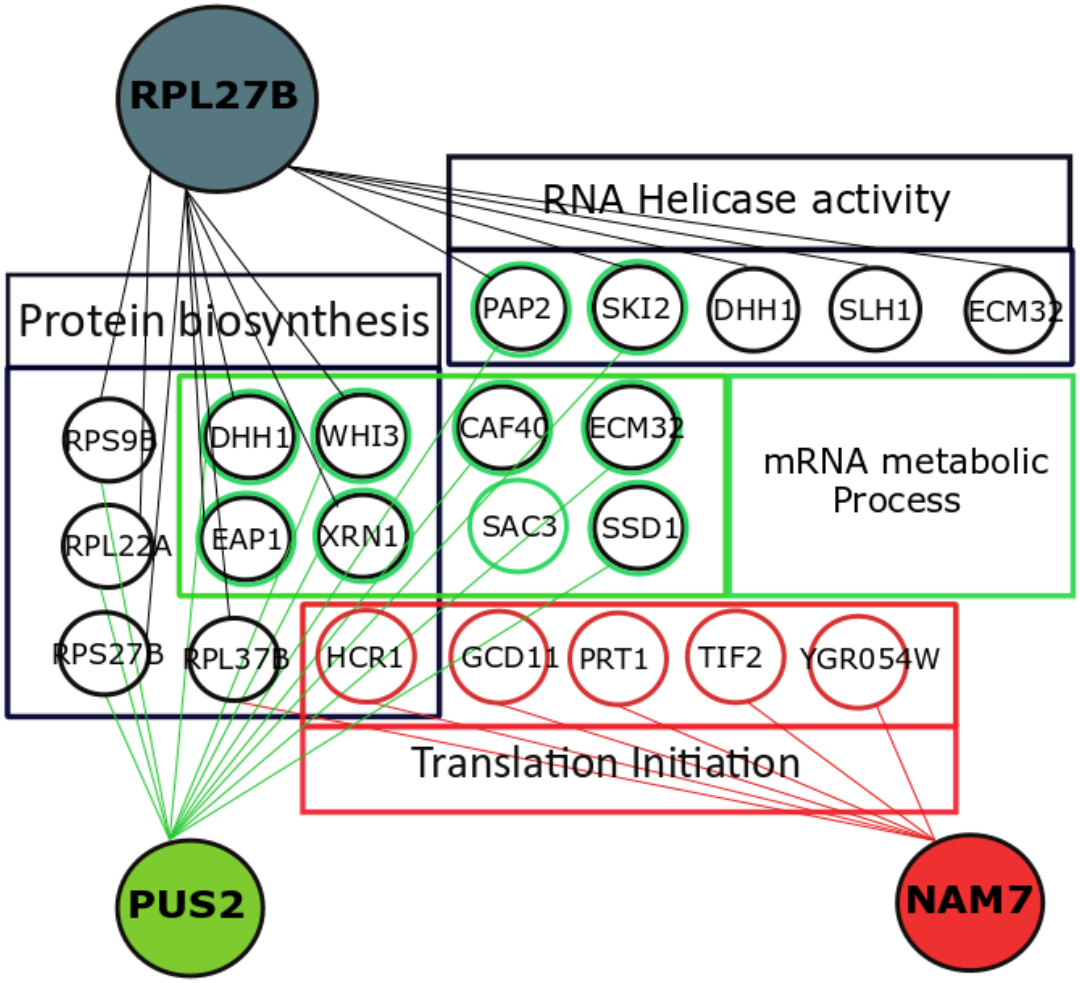


Figure 3.6. nGIs for *NAM7, PUS2,* and *RPL27B*. Our data shows a cluster of interactors involved in protein biosynthesis pathway for *NAM7* (P= 1.6e-07) *PUS2* (P= 4e-08) and *RPL27B* (P= 1.2e-05). *RPS9B*, *RPL37B*, *RPS7B*, *RPL22A*, *DHH1*, *EAP1*, *WHI3,* and *XRN1* are mutual hits shared between *NAM7, PUS2,* and *RPL27B.* Circles represent genes, lines represent nGIs identified in this study, black circles showing nGIs for *RPL27B*, green circles showing *PUS2* and red are *NAM7*.

Another form of nGIs can be studied under the sub-inhibitory concentration of drugs to investigate possible candidate genes being activated under specific conditions called conditional SGA [28,33]. We were looking for the regulation of candidate genes important in translation control under LiCl toxicity. For this experiment, we studied SGA analysis for

NAM7, *PUS2,* and *RPL27B* under the sub-inhibitory concentration of LiCl (3 mM). Shown in Figure 3.7, new nGIs were observed for our candidate genes which are involved in the translation pathway but mostly translation regulation, indicating their roles in response to LiCl toxicity in the cell. *BCK1*, *CTK1,* and *MCK1* were the mutual interactors between our genes which have been shown to be important in translational control, specifically for LiCl toxicity in our case. *BCK1*, *EAP1,* and *POP2* (also nGIs) are important in the negative regulation of translation under stress conditions. They are important in deadenylation and decapping of mRNAs to be degraded in correlation with *DHH1,* an important RNA helicase in the MAP kinase pathway to regulate the expression of specific genes [169,171–173]. On the other hand, *CTK1* is a conserved kinase that phosphorylate RPS2p, one of the components of small ribosomal subunit, affecting translation fidelity during elongation as well as phosphorylation of other translation initiation factors including eIF4A, eIF5, eIF4G, and eIF3, to improve their function as crucial factors of proper translation initiation [186,187]. It was shown that eIF4A physically interacts with cyclin-dependent kinase A (homolog of *CTK1* in plants) which is important in cell proliferation and cell cycle [80] is also a target of LiCl. Another interesting mutual hit we observed in these screens was *MCK1*, which happens to be involved in GSK-3 pathway, one of the main targets of LiCl aside from the Protein Kinase C pathway [146].

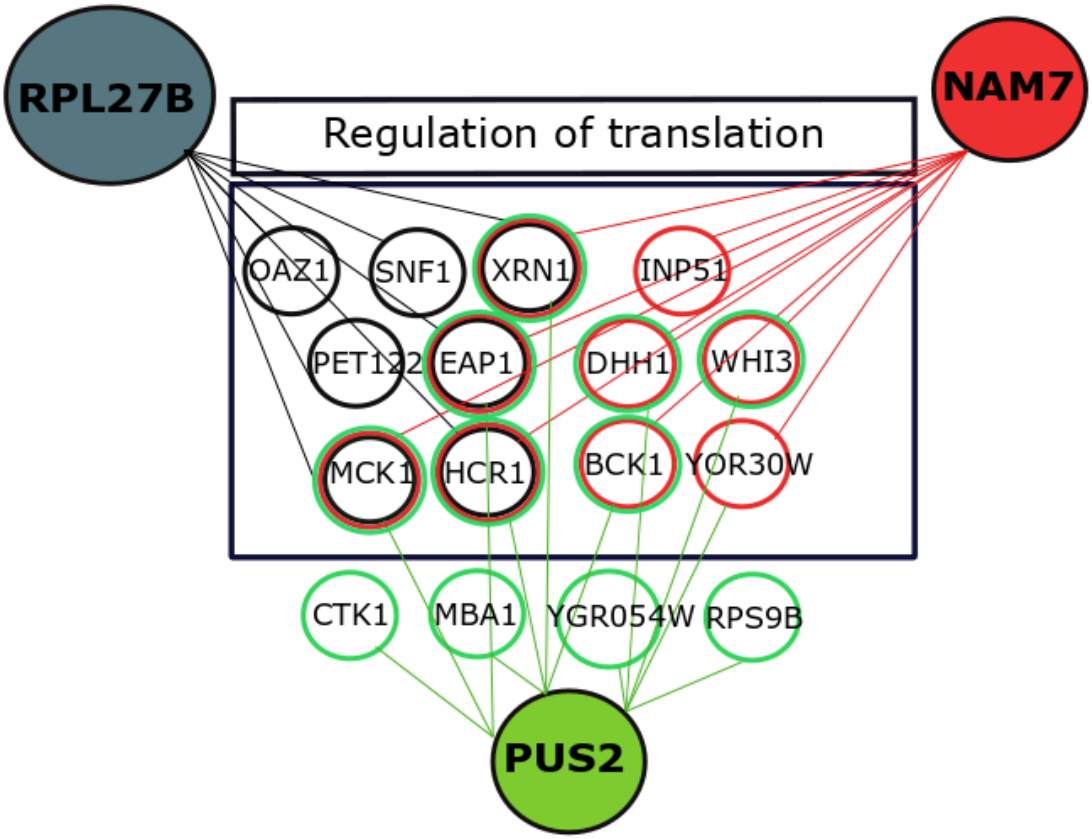


Figure 3.7. Conditional nGIs for *NAM7, PUS2,* and *RPL27B* under 3 mM concentration of LiCl. Our data shows a mutual GO term cluster of translation regulation genes, with *BCK1*, *MCK1*, *CTK1*, *EAP1* and *DHH1* as mutual nGIs between *NAM7* (P= 0.00037), *PUS2* (P= 1.1e-06) and *RPL27B* (P= 1e-06), apart from genes involved in translation (not shown). Circles represent genes and lines represent nGIs identified in this study, black circles showing nGIs for *RPL27B*, green circles showing *PUS2* and red are *NAM7*.

PSA analysis focuses on another form of interaction where over-expression of one gene compensates for the absence of another gene under drug treatment [7,31,34,168]. In our case, we treated our arrays of mutant strains with 10 mM LiCl and consequently, some showed sensitivity. Then we were able to revert LiCl sensitivity of a number of deletions by introducing overexpression plasmid of *NAM7*, *PUS2,* and *RPL27B*. We were able to find some interesting mutual hits, in which over-expression of either *NAM7*, *PUS2* or *RPL27B*

compensated for their deletion in treatment with 10 mM LiCl. These including *YGR0564W* (eIF2), *DPH2*, *EAP1*, *TMA7* and *ITT1,* which all involved in the translation pathway and posttranscriptional regulation of gene expression. All of these genes have been shown to interact with *DHH1,* a known RNA helicase with different roles in unwinding secondary structure of mRNA, mRNA decapping and translational repression [173]. *Eap1*, an eIF4E-associated protein that accelerates decapping of mRNA, is an example of a protein that correlates with *DHH1* in negative regulation of translation [171].

4. A correlation between 3'-UTR of *OXA1* gene and yeast mitochondrial translation.

4.1 Abstract

Mitochondria possess their own DNA (mtDNA) and they are capable of carrying out transcription and translation of mt-encoded ORFs. Although protein synthesis takes place in mitochondria, the majority of the proteins in mitochondria are of nuclear origin. Untranslated 3' and 5' regions of mRNAs (3'-UTR and 5'-UTR) are thought to play key roles in directing and regulating the activity of mitochondria mRNAs. Here we investigate the association between the presence of 3'-UTR from the *OXA1* gene on a prokaryotic reporter mRNA and mitochondrial translation in yeast. *OXA1* is a nuclear gene that codes for mitochondrial inner membrane insertion protein and its 3'-UTR is shown to direct its mRNA towards mitochondria. Using a *β-galactosidase* reporter gene we provide genetic evidence for a correlation between the presence of 3'-UTR of *OXA1* on mRNAs and mitochondrial translation in yeast.

4.2 Introduction

Diverse functions of mitochondria can vary from cell to cell. In addition to its function in signaling pathways and metabolism, the most predominant role of mitochondria is to produce energy for cell survival in the form of adenosine triphosphate (ATP). Mitochondrial activities are facilitated by mitochondrial proteins encoded by either nuclear DNA or maternally-inherited mtDNA [188]. Like nuclear DNA, mtDNA goes through DNA replication, transcription and translation [189]. The machineries involved in these

processes, however resemble those of prokaryotic organisms reflecting the evolutionary origin of mitochondria [190]. DNA damage, heat shock, UV radiation and other stimuli including chemotherapeutic drugs can affect mitochondria-driven apoptosis [191].

In a number of neurological disorders including Parkinson's and Alzheimer's diseases, mutations in the respiratory chain and OXPHOS system, coded by mt-DNA can lead to premature cell death [192]. Also, patients with certain mitochondrial disorders are shown to have impaired immune systems and a higher probability of infections [193]. Other mitochondrial associated disorders include diabetes and a number of cancers [194]. Targeting mitochondrial biosynthesis and limiting mitochondrial reactive oxygen species (ROS) production is thought to be a promising cancer therapeutic strategy [193]. In this context, targeting different agents including proteins, DNA, mRNA, nano-agents, and antioxidants to mitochondria has been proposed for therapeutic purposes [195,196].

Like in humans, in *Saccharomyces cerevisiae,* mtDNA carries a limited number of genes involved in gene expression, and respiratory chain, in addition to several ribosomal proteins, tRNAs, 12S and 16S rRNA. [197]. The majority of the proteins that function within this organelle are of nuclear origin and are imported to mitochondria [188]. Certain nuclear RNAs including 5S ribosomal RNA and microRNAs are also reported to enter mitochondria [198]. In a yeast genome-wide analysis 466 nuclear genes were identified that when deleted caused impaired mitochondria respiration. Human homologs of many of these genes were linked to human mitochondria disorders, indicating that yeast may serve as a suitable model organism to study mitochondria-associated human diseases [199].

It is generally accepted that the localization of certain nuclear mRNAs to the vicinity of mitochondria leads to the localized translation of these nuclear genes near mitochondria. This is followed by the import of the newly translated proteins into mitochondria [188]. An example of these mRNAs is that of the *OXA1* gene, which codes for mitochondria inner membrane insertion protein. The 3'-UTR of the *OXA1* gene was shown to direct the mRNA to the vicinity of mitochondria [200]. It is suggested that this 3´-UTR sequence serves as a binding site for certain RNA-binding proteins that, with the help of motor proteins, transport mRNAs towards mitochondria [188]. Then, *OXA1* mRNA is translated near the outer surface of the mitochondria. This is followed by the import of the newly synthesized Oxa1p into mitochondria [201]. In this way, the presence of Oxa1p in mitochondria is directly influenced by the 3'-UTR of its mRNA. However, from the current data, it is not clear if a portion of *OXA1* mRNA molecules can also enter mitochondria. The main reason for this lack of knowledge stems from technical limitations associated with purification experiments. An inherent limitation of purification procedures is that they all suffer from different degrees of co-purifying contaminants. Consequently, deriving precise conclusions from purification experiments alone is not an easy task. There is often a need for designing unique approaches to answer specific questions. *OXA1* mRNA is known to be abundant and efficiently translated in the cytoplasm of yeast, with the currently available methodologies, it is very challenging to differentiate whether a small portion of *OXA1* mRNA may also get into and translated in mitochondria.

In the current study, we investigate the 3'-UTR of *OXA1* mRNA for its possible translation in the mitochondria. We designed an approach that uses the foundations that govern mitochondrial translation as the basis to evaluate the translation of a prokaryotic reporter *β-galactosidase* mRNA inside mitochondria. An important feature of our approach is that the construct is designed to hinder the cytoplasmic translation of its mRNA. Our observations provide genetic evidence for a correlation between 3'-UTR of *OXA1* mRNA and the translation of *β-galactosidase* mRNA by mitochondrial translation machinery.

4.3 Materials and Methods

4.3.1 Strains plasmids and media

The baker's yeast, *S. cerevisiae* strain BY4741 was used in this study. The expression vector p416GALL [32] was utilized for expression analysis. This vector contains a galactokinase promoter (GALL-pro) upstream of a *lacZ* reporter gene that was cloned as an Xbal/BamHI cassette. The plasmid also contains URA3 and Ampicillin resistance (Amp) genes for selective growth of yeast and *E. coli,* respectively in selective media [33]. DH5α strain of *E. coli* was used to propagate plasmids. Standard rich (YPD) and synthetic complete (SC) media were used for growth of yeast except if specified otherwise. LB (Lysogeny broth) was used to grow *E. coli.* Antibiotics were used in the following concentrations: ampicillin, 100 μg/ml; cycloheximide, 45 ng/ml; and chloramphenicol, 1.4 mg/ml.

4.3.2 DNA manipulations

3’-UTR of OXA1 was amplified by PCR from genomic template using the following primers: 3'-YOXAlUTR-F (5’ CGCGGATCCATTAATAACAAAAAATGAATAAAGGC 3’) and 3'-YOXAlUTR-R (5’ CGCGGATCCTCCAAATGATTATTTCAAGCAATAAA 3’). The resulting PCR product was digested by BamHI and ligated into the unique BamHI site of p416GALL. Sequencing was used for confirmation. Designed 5’-UTRs of COX2 were cloned by inserting synthesized double stranded DNA into the unique XbaI site of p416GALL plasmid. The following pairs of primers were synthesized and hybridized in ligation buffer. Primers 5COXa (5’ AATAGTATTAACATATTATAAATAGACAAAAGAGTCTAAAGGTTAAGATTTATTAAAATGC 3’) and 5COXb (5’ CTAGGCATTTTAATAAATCTTAACCTTTAGACTCTTTTGTCTATTTATAATATGTT AATACTATTACGCCATGGTCAGCTTACGCCCGCCTGTTTGGCGGGCGTAAGCTGG 3’) were used for MT-C1 construct and primers 5COXDa (5’ CTAGCCAGCTTACGCCCGCCAAA CAGGCGGGCGTAAGCTGACCATGGCGTAATAGTATTAACATATAGATCTTAGACAAAAGAGTCT AAAGGTTAAGATTTATTAAAATGC 3’) and 5COXDb (5’ CTAGGCATTTTAATAAATCTTAACCTT TAGACTCTTTTGTCTAAGATCTATATGTTAATACTATTACGCCATGGTCAGCTTACGCCCGCCTGT TTGGCGGGCGTAAGCTGG 3’) were used for MT-C1X construct. Sequencing was used for confirmation. Plasmids were extracted from transformed cells via QIAprep Miniprep kit, using the manufacturer’s specifications.

4.3.3 qRT-PCR

cDNA synthesis of mRNA samples was processed according to Bio-Rad RT-PCR kit.

Quantitative PCR was performed using iQSYBR Green master-mix kit (Biorad) according to the manufacturer's instructions. qPCR amplification and detection were performed on RT-PCR cycler (ROTOR GENE RG-3000 from Corbett research). Data were analyzed using Rotor-Gene Real-Time Analysis Software 6.0.14.

4.3.4 *β-galactosidase* liquid assay

The quantitative *β-galactosidase* assay was performed using ONPG (O-nitrophenyl-a-D-galactopyranoside) method as described [30]. Cells were induced by exposure to 2% galactose for 6 hrs. As needed, cycloheximide or chloramphenicol was added to the induction media. When required yeast mitochondria were isolated using BD Free Flow Electrophoresis (FFE) system according to the manufacturer's specifications.

4.4 Results and Discussion

4.4.1 In isolated mitochondria, the activity and content of a *β-galactosidase* mRNA is correlated to 3'-UTR of *OXA1* gene

mRNA targeting is generally regarded as a component of localized translation. The 3'-UTR of *OXA1* mRNA is believed to mediate the targeting of this mRNA to the vicinity of mitochondria where the mRNA is translated and subsequently imported into the mitochondria [202]. *OXA1* codes for a mitochondria inner membrane protein that is thought to play a role in membrane binding of ribosome and insertion of newly synthesized polypeptides into mitochondrial inner membrane [190]. Also, there are examples of numerous RNA molecules that are imported into mitochondria in different organisms. Various mitochondrial tRNAs are encoded by the nuclear genome and subsequently

targeted into mitochondria [130]. More recently, a 20 nucleotide sequence from the *H1* RNA, the RNA component of RNase P enzyme, was shown to mediate the import of fusion tRNA and mRNA molecules into mitochondria and rescuing phenotypic defects in two different human cell lines caused by defective tRNAs [195]. Since *OXA1* 3'-UTR is shown to target mRNAs to the vicinity of yeast mitochondria, we asked the question if this 3'-UTR might also direct the mRNA into mitochondria. For this purpose, we designed a specific approach that utilized the foundations that govern mitochondrial translation and architected a distinct prokaryotic reporter *β-galactosidase* construct. This construct, termed MT-C1 for Mitochondrial Translation Construct 1, is designed to extensively hinder cytoplasmic translation of its reported mRNA and enhance its translation within mitochondria (Figure 4.1). At its 3'-end, MT-C1 carries *OXA1* 3'-UTR. It's 5'-UTR is designed to impede cytoplasm translation by including the following elements. 1. A strong inhibitory secondary structure with an estimated ΔG value of -28 kcal mol^{-1} previously shown to prevent the advancement of 40S ribosomes along mRNA and thereby inhibiting translation [203]. The purpose of this structure is to prevent the scanning of the cytoplasm ribosomes from 5'-CAP towards the start codon. 2. An early initiation start codon next to a consensus Kozak sequence. The presence of a strong Kozak sequence will promote an out-of-frame cytoplasmic translation initiation for those ribosomes that may escape the inhibitory structure. These ribosomes will start translation from the out of frame AUG codon. And 3. consecutive premature stop codons downstream of the early start codon. These stop codons can terminate unwanted translations mediated by its upstream region. The mitochondrial translation is reported to require a consensus translation initiation

sequence 5'-UAUAAAUA-3'. To promote mitochondrial translation, 5'-UTR from *COX2* mRNA, which contains the mitochondria consensus translation initiator sequence, was incorporated upstream of the AUG start codon of a prokaryotic *β-galactosidase* reporter gene. *COX2* codes for cytochrome C oxidase subunit II, the terminal member of the mt inner membrane electron transport chain and is translated in mitochondria [204]. It is one of the major mitochondrial mRNAs and has a well-characterized mitochondrial 5'-UTRs and the shortest (54 nucleotides) reported [204,205]. A second construct termed MT-C2 was designed as a control. It contains all the features of MT-C1 with the exception that it lacks the 3'- UTR of *OXA1* at its 3'-end.

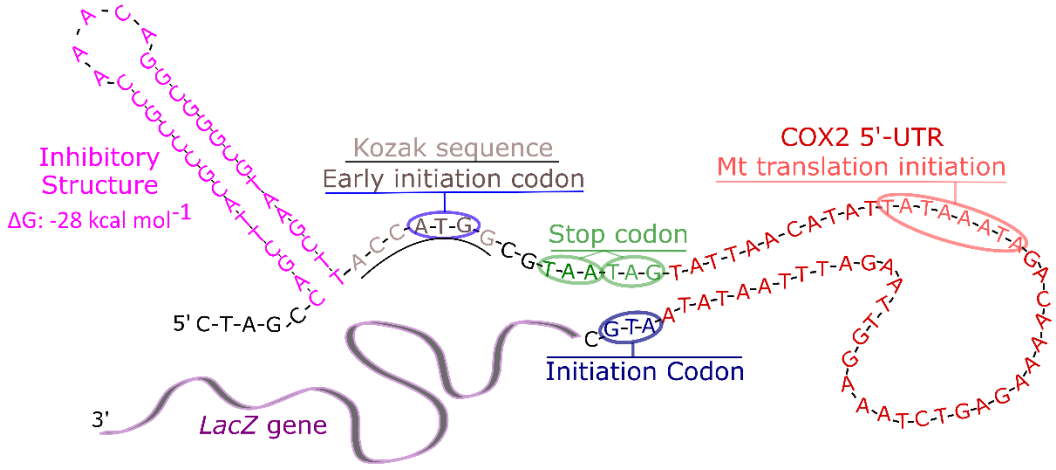


Figure 4.1. Schematic representation of the *β-galactosidase* mRNA designed to limit cytoplasmic translation. Various features of the 5'-UTR for this mRNA are shown in different colors. Elements designed to limit cytoplasmic translation include an inhibitory structure, an early initiation codon within Kozak sequence and early tandem stop codons. *COX2* 5-UTR mediates mitochondrial translation. Figure created using Inkscape.

MT-C1 and MT-C2 were used to transform yeast cells. Mitochondria were isolated from the transformed cells and the *β-galactosidase* mRNA content, as well as *β-galactosidase*

activities of the isolated samples, were measured (Figure 4.2). Represented in Figure 4.2a, *β-galactosidase* mRNA analysis using RT-qPCR indicated that mitochondrial mRNA content for those mRNAs lacking *OXA1* 3'-UTRs (MT-C2) is approximately reduced by 90% in comparison to those carrying *OXA1* 3'-UTR (MT-C1). Similarly, *β-galactosidase* activity measurement showed an approximately 85% reduction for mRNAs lacking *OXA1* 3'-UTR.

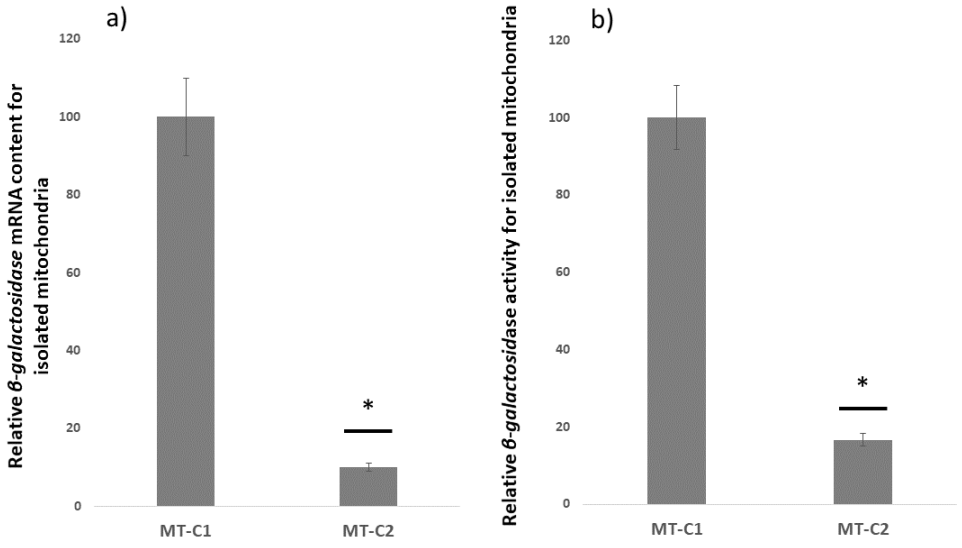


Figure 4.2. mRNA content and *β-galactosidase* expression analysis of isolated mitochondria from different yeast strains. a) RT-PCR analysis indicated that in the absence of *OXA1* 3'-UTR (MT-C2) *β-galactosidase* mRNA content is significantly reduced in isolated mitochondrial. The values are normalized to that of the MT-C1 construct that carries *OXA1* 3'-UTR. *PGK1* mRNA was used as a control and all values are related to that. Each experiment is repeated at least 3 times. Error bars represent standard deviation. b) *β-galactosidase* activity of isolated mitochondria is highly reduced in the absence of *OXA1* 3'-UTR (MT-C2). Values are normalized to that for MT-C1 set at 100. * represent statistically significant results (*P*-value ≤ 0.05) compared to the WT. Each experiment is repeated at least three times. Error bars represent standard deviation.

In these experiments, MT-C2 may represent the background levels of mRNA and *β-*

galactosidase activity. However, because of the limitation associated with all purification-based assays, it remains possible that the observations we made here are a result of the co-purification of contaminants. Similarly, we have gone to length to ensure limited cytoplasmic translation from the designed constructs. It is unlikely, but still possible that the observed translation might stem from cytoplasmic translation only. Consequently, further investigation is needed.

4.4.2 Observed *β-galactosidase* activity contains signatures of mitochondrial translation.

To further study the correlation between the translation of mRNAs carrying *OXA1* 3'-UTR and mitochondrial translation, and to indicate that it is, in fact, mitochondrial translation which is mainly detected here and not cytoplasmic translation, we designed a control construct termed MT-C1X. This new construct is identical to MT-C1construct with the exception that its 8 nt *COX2* translation initiation signal that promoted mitochondrial translation within 5'-UTR of MT-C1 was deleted. Consequently, if we were to detect cytoplasmic translation in our assays (for example, through co-purification as a contaminant) we expect relatively the same level of translation for MT-C1 and this new control construct MT-C1X. However, if we were to observe mitochondrial translation, the *β-galactosidase* level observed for MT-C1X should be similar to that for MT-C2. Illustrated in Figure 4.3a, it was observed that the *β-galactosidase* activity of isolated mitochondria from strains carrying mRNAs lacking mitochondrial translation signal, MT-C1X, was reduced by approximately 90% from that observed for the intact parent construct with

the mitochondrial translation signal (MT-C1), and to levels comparable to MT-C2 (the construct lacking *OXA1* 3'-UTR). In this case, the level of translation associated with MT-C1X may represent co-purified contaminations and the background level of translation, including cytoplasmic translation within the vicinity of mitochondria, a phenomenon that has been observed in previous studies for the natural *OXA1* mRNA [200–202]. This observation provides evidence that *β-galactosidase* observed for MT-C1 is connected to mitochondrial translation and not as a result of co-purification artifacts.

Next, we used antibiotics that specifically targeted different modes of translation (cytoplasmic vs mitochondrial). As indicated above, yeast mitochondrial translation resembles the prokaryotic mode of translation whereas cytoplasmic translation represents that of eukaryotes. To further investigate if the *β-galactosidase* activity mediated by the MT-C1 construct is primarily influenced by the mitochondrial (prokaryotic) mode of translation, we utilized antibiotics thought to be specific to one mode of translation and not the other. To this end, we utilized cycloheximide and chloramphenicol. Cycloheximide is a eukaryotic translation inhibitor [160]. It interferes with the translation elongation step by binding to the E-site of the eukaryotic ribosome. Chloramphenicol is a bacterial translation inhibitor. It disrupts peptidyl transferase activity of the ribosome during prokaryotic translation. The presence of cycloheximide in the media reduced the level of *β-galactosidase* activity mediated by MT-C2 and MT-C1X by approximately 83% and 95%, respectively (Figure 4.3b). This is in comparison with an approximately 46% reduction for MT-C1. These observations suggest that translation mediated by the MT-C1 construct seems to be more resistant to cycloheximide. In

contrast, when the growth media was supplemented with chloramphenicol, *β-galactosidase* produced by MT-C2 and MT-C1X was reduced by approximately 15% and 12% indicating that they are not very sensitive to this concentration of chloramphenicol (Figure 4.3c). A reduction of 44% was observed for MT-C1 suggesting that translation mediated by MT-C1 construct appears to be more sensitive to chloramphenicol.

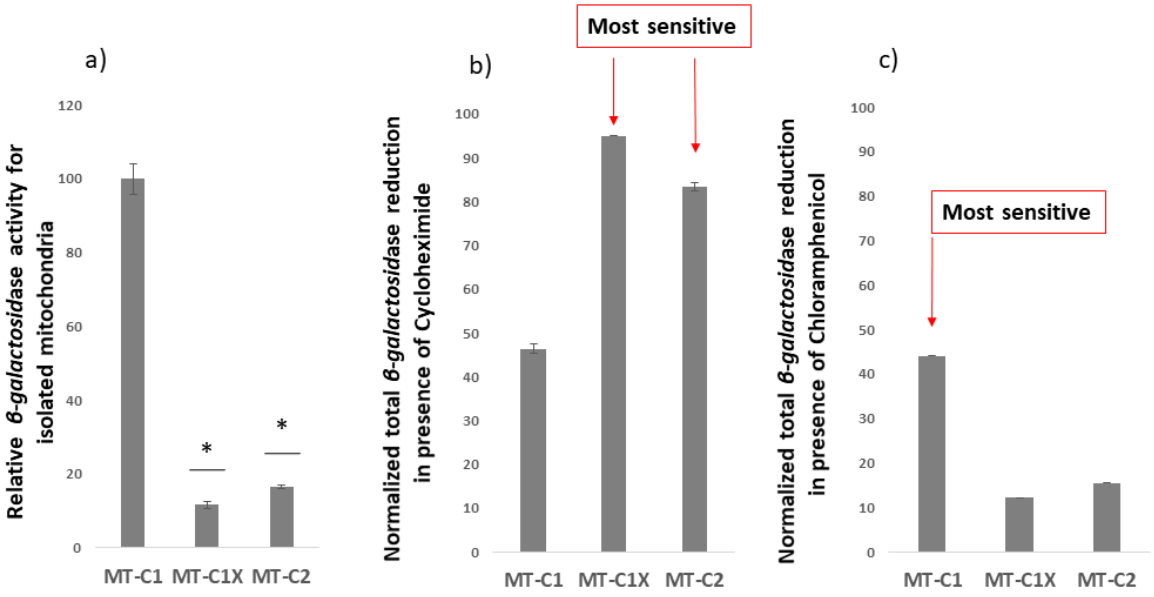


Figure 4.3. Analysis of relative *β-galactosidase* activity analysis a) *β-galactosidase* activity of isolated mitochondria is highly reduced in the absence of *COX2* mitochondrial translation initiation signal MT-C1X in comparison to MT-C1 construct that contains the translation initiation signal. b) In the presence of 45 ng/ml of cycloheximide, a cytoplasmic translation inhibitor, *β-galactosidase* mediated by MT-C1X and MT-C2 showed the most sensitivity. c) In contrast, when 1.4 mg/ml of chloramphenicol, a prokaryotic translation inhibitor, was added *β-galactosidase* activity mediated by MT-C1 showed the most sensitivity. The values in a) are related to the value of MT-C1 and values in b) and c) are normalized to the translation of the same construct in the absence of the corresponding translation inhibitory compound. * represent statistically significant results (*P*-value ≤ 0.05) compared to the WT. Each experiment is repeated at least 3 times. Error bars represent standard deviation.

The observations made in the current study provide genetic evidence for a correlation between the 3'-UTR of the *OXA1* gene on a reporter mRNA and yeast mitochondrial translation. In addition to the reported ability of 3'-UTR of *OXA1* gene to direct mRNAs to the vicinity of mitochondrial, it seems possible that this 3'-UTR might also direct mRNAs into the mitochondria where the mRNAs can get translated by the mitochondrial translation machinery (Figure 4.4). The suggested model (Figure 4.4) should be considered at a proposal level. Detailed biochemical assays should be employed to further study this proposed model. In future, it would be of interest to further study the relationship between this 3'-UTR and mitochondria using other reporter genes and in other organisms including humans. The potential ability of *OXA1* 3'-UTR to direct an mRNA into human mitochondria could have important implications for human health. A number of human genetic diseases are linked to defects in mitochondria encoded genes or those nuclear genes whose products are directed into mitochondria [206,207]. In this context, the ability to direct mRNA into mitochondria could provide an appealing therapeutic opportunity.

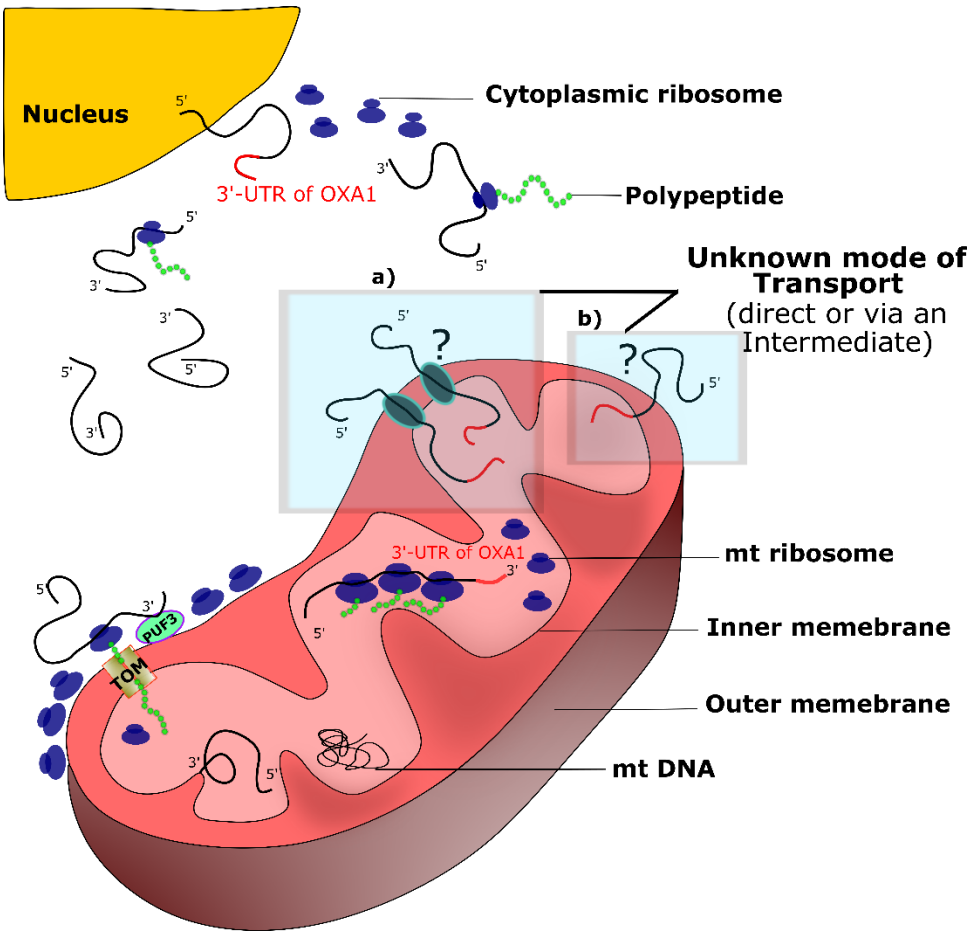


Figure 4.4. A model for the role of 3'-UTR of the *OXA1* gene. In addition to its reported activity in recruiting mRNAs to the vicinity of mitochondria, 3'-UTR of the *OXA1* gene may also direct mRNAs into mitochondria via a currently unknown mechanism. The majority of mitochondrial proteins are of nuclear origin and are imported into mitochondria after translation. The 3'-UTR of yeast *OXA1* mRNA can direct mRNAs to the vicinity of mitochondria via interaction with mitochondria outer surface protein Puf3. The newly translated polypeptides, near the vicinity of mitochondria, are directed into mitochondria using general translocase of the outer membrane (TOM) complex. Here we propose that the 3'-UTR of *OXA1* mRNA may also mediate the entry of mRNAs into mitochondria where they are translated by mitochondrial translation machinery. Figure created using Inkscape.

5. Translation regulation of highly structured 5'-UTR constructs.

5.1 Abstract

Helicases are important for the translation of structured mRNAs. Growing evidence suggests that certain helicases affect the expression from specific mRNA structures. To further investigate proteins factors that affect the translation of specific mRNAs with distinct structured 5'-UTRs, we designed four yeast reporter constructs. In these constructs, translation of a *LacZ* reporter cassette is placed under the control of four different structured mammalians 5'-UTRs. Yeast knockout collection was used to identify yeast gene knockouts that show reduced expression. This resulted in the analysis of approximately 75,000 yeast colonies for *LacZ* expression. In this way, we identified a number of protein factors that seem to be specific to one or more structures. Interestingly we did not identify any protein factors that were linked to the translation of all 4 structures.

5.2 Introduction

In mammalian cells, *Dhx29* and *Ddx3* play important roles in the translation efficiency of mRNAs with strong secondary structure ΔG< -40 Kcal/mol or long 5'-UTRs; for the less structured mRNAs however, they seem to have little or no effects [90,91]. Generally, it is accepted that eIF4A is the main RNA helicase in the translation pathway that participates in unwinding secondary structures on most mRNAs. However, growing evidence suggests

that the scanning of more structured mRNAs requires the recruitment of specific helicase enzymes [91,104]. The control and regulation of additional helicases seem to be connected to specific group of mRNAs with structured 5'-UTRs mainly involved in different cellular processes including regulation of growth, proliferation, and apoptosis [90]. Consequently, it is important to further study additional mRNA helicases and the factors that contribute to their activity as well as their regulation and control. Molecular signals that mainly regulate translation are MAPK and mTOR signals [6].

Some helicases seem to be specific to certain structures. Cell under stress regulate translation of mRNAs needed for maintaining homeostasis or apoptosis. One way to control their translation is through activation of specific helicases specific for unwinding their highly structures [135]. It is known that retroviruses carry highly structured region in their 5'-UTR RNA that are crucial for their translation. These RNA structures are found to function as PCE rather than IRESs and the translation of the mRNAs with highly structured UTRs is modulated through cap-dependent protein synthesis [102]. This was observed in HIV-1 replication where the retrovirus requires RNA helicase A (RHA) for proper translation in the host cell for the recognition of a conserved PCE [102]. *DDX3*, *DED1* in yeast, and RNA helicase A in coordination with eIF4A facilitate ribosomal scanning by unwinding the secondary structure or remodeling ribosome for a better scanning [181].

Similarly, it was recently shown that G-quadruplex (rG4) structures on the 5'-UTR of certain human mRNAs regulate gene expression in a post-transcriptional manner [208]. Helicases *DHX36* and *DHX9* were shown to affect the translation of mRNAs with rG4 signatures.

To further study helicases and protein factors that may regulate the activity of helicases as well as to identify additional ones that might be specific to a given structure, we designed four expression constructs where a reporter mRNA is under the translational control of different mammalian hairpin structures. Yeast gene knockout collection was transformed with these constructs to screen for gene deletions that reduce the activity of the reporter *LacZ* cassette.

5.3 Materials and Methods

5.3.1 Media and Strains

Yeast deletion set (mating type "a", (BY4741)) was used for large-scale investigation for modified-SGA to transform the constructs into mutants and to perform lift assay. Yeast mating type "α" (BY7092) was used for plasmid transformation to deletion set [51]. DH5α, *E.coli* strain, was used to generate plasmids. Standard rich (YPD 1% Yeast extract, 2% Peptone, 20% D-glucose) and Synthetic Complete (SC) media were used to grow yeast. LB (Lysogeny Broth) was used to grow *E.coli*. 2% agar was used for all solid media. –URA media was used as a marker for selection of plasmid in yeast and LB with Ampicillin (50 mg/ml) for selection in bacteria. Geneticin, (G418), (200 mg/ml), Canavanine (100mg/ml) were used for selective growth media in modified-SGA technique.

5.3.2 Plasmids

P416 plasmid with a *LacZ* expression cassette and a gal promoter was used as our background plasmid for cloning and as a control. To generate reporter *LacZ* mRNAs under the translational control of complex RNA structures, four different fragments were cloned

upstream of the *LacZ* mRNA in p416 construct using *XbaI* restriction site. In this way four expression constructs were designed as follows: pTAR construct contains the 5'-UTR of *HIV-tar1* gene, known to have PCE elements detected by RNA helicase A [102] (5'GGGTTCTCTGGTTAGCCAGATCTGAGCCCGGGAGCTCTCTGGCTAGCTAGGGAACCCACTGC TTAAGCCTCAATAAAGCTTGCCTTGAGTGCTTCAAGTAGTGTGTGCC 3'), pRTN that contains the 5'-UTR of *FOAP-11* gene, a conserved hairpin structure in vertebrates [164] (5'GGGATTTTTACATCGTCTTGGTAAAGGCGTGTGACCCATAGGTTTTTTAGATCAAACACGTCT TTACAAAGGTGATCTAAGTATCTC 3'), 5'-UTR of *BCL3*, a transcription activator, which has been shown to be over-expressed in β-Cell lymphoma3, probably due to this highly structured mRNA [209] (5' CCGTCCCCGGCGGCCCCATGCCCCGATGCCCCCGCGGGGGCC 3') and a sequence with two hairpins (5' CTCCCCCCCCCAGAAGTAGTGTTTTTTGGAGGCCTAGG CTTTTGCAAATGACCGAGTCCGTCCCCGGCGGCGGGGGGGAT 3').

Yeast plasmid extraction was performed using yeast plasmid miniprep kit (Omega Biotek®) and *E. coli* plasmid extraction was carried out using GeneJET plasmid miniprep kit (Thermofisher® and Bio-Basics®) according to the manufacturers' instructions.

5.3.3 DNA transformation

Plasmid transformation to yeast was performed using the Lithium Acetate method [210] and through competent cells described by Inoue et al, [211]. Modified SGA technique was used to systematically transform constructs into yeast deletion library [212].

5.3.4 *β-galactosidase* Assay

White/Blue Assay or Lift Assay was performed using the X-gal compound to determine the translation activity of *LacZ* reporter gene [213]. The quantitative *β-galactosidase* assay was performed using ONPG (O-nitrophenyl-α-D-galactopyranoside) [32,158].

5.4 Results and Discussion

To investigate candidate genes involved in translation regulation of mRNAs with highly structured 5'-UTR, cloned plasmids pTAR, pRTN, pB-cell, and p2hair, containing 5'-UTR of *HIV1-tar* gene, 5'-UTR of *FOAP-11* gene, 5'-UTR of *BCL3* and with 2 hairpins respectively, along with p416 (as a control structure with no inhibitory structure) were systematically transformed into yeast gene deletion mutant array. This was made possible through a modified SGA technique originally designed to study GIs [51,212]. Plasmids were first transformed into "α" mating type strain. The transformed strains were then systematically mated to deletion library (nearly 5000 mutants) of "a" mating type. Diploid strains were then subjected to different rounds of selection to select haploid mutants carrying the plasmid with mutants in "a" mating type. This resulted in a total of 75,000 transformations.

To determine the translation activity of *LacZ* cassette in different mutants carrying target constructs we performed a *β-galactosidase* Lift Assay. In this assay, if the reporter gene is expressed and efficiently translated, then cells would turn blue after the addition of an X-gal solution (Figure 5.1). White colonies represent mutant strains that do not efficiently translate the structured mRNAs and hence represent potential hits. Potential hits are

considered hits only if they were capable of producing *β-galactosidase* with the control construct (p416). In this manner, hits can in fact express the *β-galactosidase* gene but only if it does not carry an inhibitory structure. Lift assays were repeated three times for each plasmid and hits observed at least two times were selected.

In our experiments, it is expected that the deletion of genes that are associated with detecting and unwinding the hairpins would show less translation activity. Or to identify pathways that are involved in regulation of highly structured mRNAs that when mutated, translation control is inhibited. These data, combined with our data from the original construct with inhibitory structure (p281-4) that was used in previous chapters can illuminate some information about candidate genes responsible for resolving the secondary structure of these hairpins. Interestingly, there was no common factor that seems to influence the translation of all their constructs highlighting the diversity of requirements for different mRNAs. In light of our current investigation, it seems that different mRNA structures may have different protein factors required for proper translation.

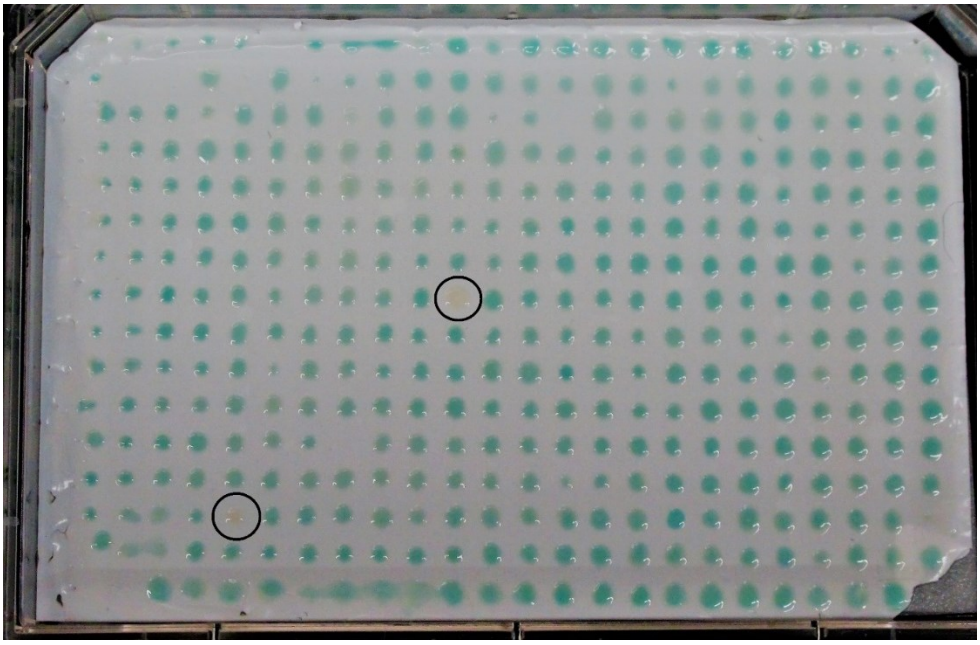


Figure 5.1. An array of deletion set, showing yeast mutant strains carrying a plasmid with a *LacZ* cassette reporter gene. The strains were lifted on a nitrocellulose paper, burst in liquid nitrogen and treated with 2% X-gal. Production of *β-galactosidase* was visible by the presence of a blue color. White colonies (shown with black circles) are those that did not produce *β-galactosidase.* They represent gene deletions where, presumably, the hairpin structures inhibited the translation of *β-galactosidase* mRNA.

To quantify translation efficiency and confirm the hits from the previous step, the liquid *β-galactosidase* assay was performed using the ONPG approach [158] in triplicates. To this point, 118 candidate genes in a total from our four constructs were selected to be analyzed and investigated further. Table 5.1 shows the candidate genes for each plasmid. A number of candidates were found to be common to "two hairpin" structures. For example, there are four common genes between pTAR and p2hair. With a P-value of 1.2 e-5 is seems that this overlap may not be by chance alone. Consequently, there might be a statistically significant overlap between the requirements for certain hairpins. Further

analysis is required to establish a relationship between these protein factors and the structure of the hairpins.

Our preliminary GO term enrichment analysis of the hits revealed that many of the hits are involved in the regulation of signaling pathways, negative regulation of the nucleobases-containing compound metabolic process, regulation of cell communication and carbohydrate kinase activity. Considering the fact that mRNA helicases actually are involved in most processes related to mRNA metabolism including transcription, splicing, mRNA export, decay and other pathways like ribosome biogenesis and translation we can propose that network analysis of our candidate genes represent a broad range of these pathways specially in translation control and signaling pathways under stress.

At this point, we have finalized the analysis of approximately 75,000 yeast colonies and generated preliminary screening data. Additional complimentary experiments are required to narrow down possible candidate genes involved in unwinding these structures. For example, although our experience has shown that most of the hits generated in this fashion identify genes that target translation, it remains a possibility that many of them may influence other processes including transcription and mRNA stability.

Table 5-1. Candidate genes that seem to influence the expression of different *β-galactosidase* constructs pTAR, pRTN, pB-cell and p2hair. Selected hits were chosen based on the white color performing lift assay (repeated three times and was observed at least twice in the repeats) and confirmed by liquid *β-galactosidase* assay. The red cells are representing common genes between different constructs and yellow cells represent those that were identified in our previous screenings.

pTAR	pRTN	pBcell	p2hair
MFT1	GAL3	MFT1	FRT1
PEX7	RTT106	FRT1	GAL3
RRN10	URA2	SEC22	MFT1
THP2	GUT2	YNL140C	PEX7
YNL140C	IML3	IRC3	RRN10
PUB1	MRX1	HSP31	RTT106
PRM2	PHO87	KEX2	SEC22
PPM1	PPR1	LSB3	THP2
RPP1A	REV7	GPD2	URA2
SCS3	RPL23A	NCA2	YNL140C
SKI2	RRD1	NVJ2	NAM7
URA4	CSN12	PEX31	PUS2
YMR226C	SAP4	PEX9	SET6
YOR072W	SBH2	PIR3	NSI1
CMP2	Ski5	PPG1	CSE2
HSL1	SLI15	PTC1	FLX1
MET18	SMI1	RGT1	FUN9
	SWH1	RPS6A	RSC2
	TAE1	HNM1	GIM3
	CEM1	SIF2	SAC7
	YBR042C	SKO1	HEF3
	YIL067C	STC2	SEM1
	YJL045W	TDH3	CIN5
	YJL163C	WTM1	SKS1
	YJR084W	YCL042W	SWI4
	YLR114C	YDR210W	SWI6
	YRO2	YKL133C	TEP1
		YLR404W	LSM6
		YMR018W	TOP1
		FET3	MIX14
		YPR091C	YDR338C
			YMR289W

			YNL003C YNL128W MRN1 YNL153C YNL171C YPL034W YPL184C

6. Conclusion

6.1 Concluding Remarks

One of the main objectives of our research laboratory is to investigate the novel function of genes in various pathways. This is often done using systems biology approaches and through large-scale screening followed by complementary experiments. Discovering novel functions for genes, complexes and pathways can lead to a broader understanding of how a cell functions as a living system. For our investigations, we focus on the baker's yeast, *S. cerevisiae*. As a tool, this model organism not only facilities high throughput experiments developed for molecular genetic studies, but also serves as an asset because of its considerable homology with the human genome and with genes causing certain complex diseases in humans. With the availability of different arrays including yeast mutant array of nearly 5000 non-essential genes, over-expression array, and GFP-tagged yeast ORF library along with different bioinformatics software for analyzing, visualizing and extracting meaningful information from large-scale data, yeast has become a good candidate for functional genomics studies [8,24,54].

Translation pathway, a conserved pathway in eukaryotes and prokaryotes, is responsible for the production of functional and structural proteins in the cell. Proteins are considered as structural and transportation molecules in the cell along with their enzymatic activities which are all crucial for cell survival and cell maintenance. Malfunction or dysregulation in this pathway can cause cell death and give rises to different types of complex diseases in humans. Diseases like cancer, neurodegenerative diseases and cognitive disorders have

been linked to failure in proper function of translation or in translation regulation processes. Hence, this pathway has been the target of therapeutic researches for a long time. Translation initiation is often considered as the most rate-limiting step in gene expression and is generally regulated through the cooperation of numerous eukaryotic initiation factors, mRNA properties, and ribosomes. Mutations in initiation factors have been linked to important diseases. For example, it is observed that in B-cell lymphoma cells, translation regulation becomes defective and causes higher translation rate of oncogenic genes specifically the ones with highly structured mRNAs [209] proposing to be due to over-expression of eIF4B an activator of eIF4A, which causes a higher rate of translation for oncogenic genes. Another example is in HIV infection where the virus uses the host translation machinery to specifically regulate its own genome. The HIV virus genome consists of mRNAs with highly structured 5'-UTR detectable by specific RNA helicases like RNA helicase A [102].

RNA helicases are responsible for unwinding secondary structures of mRNA and in some cases remodeling of the 40S ribosomal subunit for better scanning of mRNA through the translation process. They also play roles in transcription, mRNA splicing, mRNA export and storage, translation activation/inhibition and mRNA decay pathway [98,214,215]. In addition to helicase activity RNA helicases may have other biochemical activities including RNA clamping, double-stranded RNA destabilization, dissociation of proteins from RNAs and strand annealing [216]. eIF4A, the main known helicase of translation pathway, is a component of eIF4F complex and is known to regulate translation of most mRNAs in the cell and is responsible for resolving 5' cap structure, whereas other RNA helicases are

thought to be involved in recognition and unwinding of mRNAs with highly structured 5'-UTRs like *DED1* and its close homolog *DBP1* in human. It is proposed that different types of mRNAs, based on their secondary structure at 5'-UTR, demand different types of helicases and associated factors to regulate their translation [217].

The structure of mRNA 5'-UTR is a feature that can regulate the post-transcriptional expression of different mRNAs. This regulation, at the translation level, can be in a cap-dependent or cap-independent manner. In cap-dependent regulation, usually remolding or unwinding of RNA structures happens with the help of different RNA helicases or other RNA interaction processes (for example with sRNAs) to regulate scanning. In a cap-independent manner, the regulation is through direct recognition of RNA structures like IRES or PCE elements and mRNA modifications [217]. New findings show that the structure of mRNA, regardless of its negative folding free energy, can recruit translation machinery even more efficiently than those with no hairpin structure [217].

Although much has been learned about the function of known RNA helicase involved in translation and other pathways, there is still much to learn about the novel function of different factors that can influence the translation of structured mRNAs. mRNA structure is one of the components that determine the rates and regulation of mRNA translation. In a recent study using ribosome profiling under inhibition of eIF4A by different drugs like Silvestrol and Rocalglamide, it was observed that the translation of mRNAs with CGG motifs was selectively reduced but not mRNAs with long UTRs [217]. Another study that investigated 5'-UTR of mRNAs through deletion of eIF4A followed by ribosome profiling

and RNA sequencing revealed U-rich and GA-rich motifs on mRNAs as important regulators of translation [218,219].

In the current thesis, we investigated novel yeast gene functions that affect the translation of structured mRNAs. For this, we screen the library of yeast nonessential gene deletion for those genes that affect the translation of a structured reporter construct.

Using our original structured reporter construct p281-4, 24 candidate genes were identified. Among these nine candidate gene deletions including *yta6Δ*, *ypr096cΔ*, *nam7Δ*, *pus2Δ*, *rpl27bΔ*, *rps23bΔ*, *srs2Δ*, *ypr089wΔ,* and *hal1Δ* showed the most sensitivity to LiCl treatment (10 mM). Further, we showed that over-expression of the mentioned genes can revert LiCl toxicity in the cell, in the same that over-expression of eIF4A compensated LiCl sensitivity [148]. Follow up experiments performed to confirm that this was due to translation deficiency rather than reduction at the transcription level.

In the next phase of our investigation, to further study different protein factors required for the translation of specific structured mRNAs we designed additional. Four constructs named pTAR, pRTN, pB-cell, and p2hair were generated for this purpose. The early screening revealed some mutual hits with our previous data, including *NAM7*, *PUS2*, *IRC3*, *SET6,* and *PUB1*. Certain overlaps were observed between protein requirements for different structures. However, no common protein factor was found in all the screens. This further highlights that protein requirements for translation of different mRNAs are in fact variable. Further study is required to confirm our preliminary results. We expect most of these genes to indirectly affect the translation. Consequently, future studies can

focus on generating double gene deletions to illustrate the intermediator gene(s) or pathway(s) that are influenced by these genes. Some of these genes may also have helicase activities. In this context *in vitro* assays designed to study the activity of helicases will prove to be useful.

In another study, we used translation in mitochondria as a strategy to investigate the ability of a specific 3'-UTR sequence to direct a reporter mRNA into yeast mitochondria. Our constructs were designed based on 3'-UTR sequence of *OXA1*, known to direct its mRNA to the vicinity of mitochondria. Previous studies show that Oxa1p is produced in the vicinity of mitochondria and then transferred into mitochondria [190]. Here we aimed to see if some of these mRNAs can get into mitochondria directly. Our observations provided genetic evidence for the import of a reporter *β-galactosidase* mRNA into mitochondria that are mediated by the 3'-UTR of the *OXA1* gene. Malfunction of different mitochondrial proteins is linked to various diseases. Consequently, the ability of an RNA sequence to direct mRNA into mitochondria might have therapeutic uses.

In this thesis, we investigated the activity of five genes for their novel involvement in the translation of structured mRNAs. We also performed a directed large scale screening for similar gene function and in doing so observed dozens of new functions for previously investigated genes. A conclusion that we can draw from these observations is that there seems to be much to be learned for the translation of specific mRNAs mediated by specific structures. It now seems that these structures might play more important roles than previously thought. Similarly, the involvement of additional genes in translation regulation also suggests a higher degree of complexity for translation regulation mediated

by mRNA structure. In this thesis, we only focused on certain structures. Expanding the diversity of these structures may help us discover additional gene functions related to the translation of structured mRNAs. In this context, it is worth noting that by no means are the screens we performed exhaustive. Consequently, there remains a number of additional factors that might be identified using additional and improved screens. For example, our screens only focused on non-essential genes. Including conditional knockout essential gene array into future screens can provide additional information. Similarly, our current screens are based on negative selection where the deletion of a target gene reduces the ability of translation from a structured mRNA. Future screens can focus on positive selections were the over-expression of a target gene may increase the translation of a structured mRNA.

The discovery of so many new gene functions in the current thesis also highlights the need for additional functional genomics studies. The current screen revealed dozens of potential new gene functions related to one specific feature of translation. We expect that the development of new functional genomics screens with different areas of focus can mediate the identification of additional gene functions for other aspects of translation and translation control as well as other cellular processes. The discovery of additional functions for genes can help us better realize the biology of a cell as a system and its mechanism of responses to internal and external stimuli.

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8. Appendix

8.1. Additional data for chapter 2 and 4

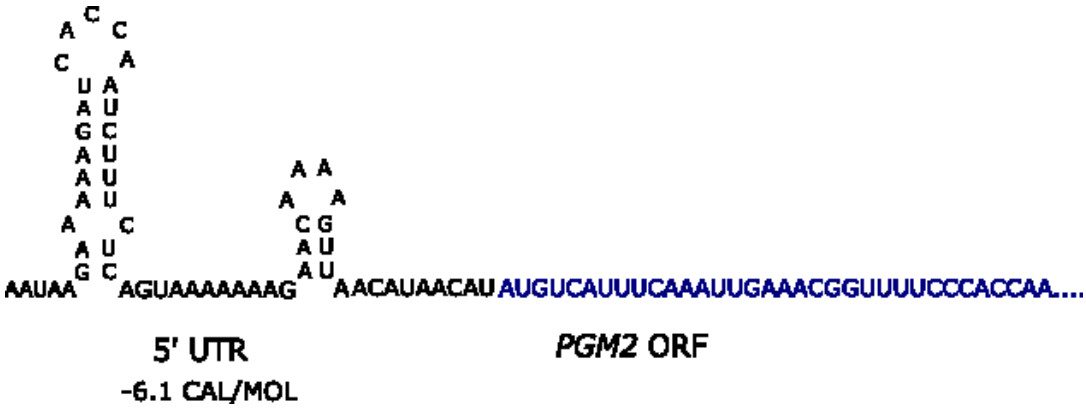


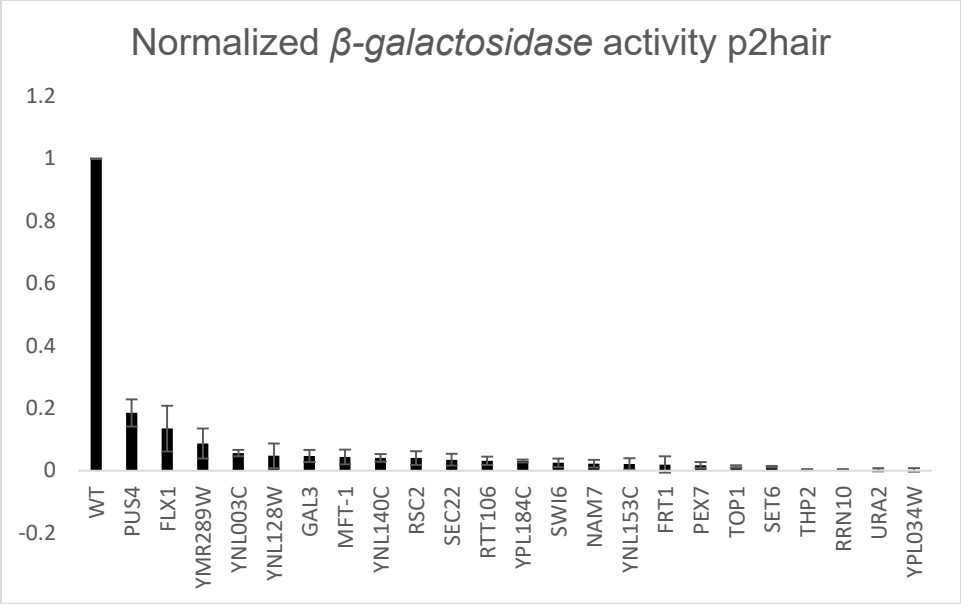
Figure 8.1. The secondary structure of *PGM2* 5'-UTR. Unlike most yeast ORFs, the 5'-UTR of *PGM2* is thought to be structured (Tuller et al., 2009).

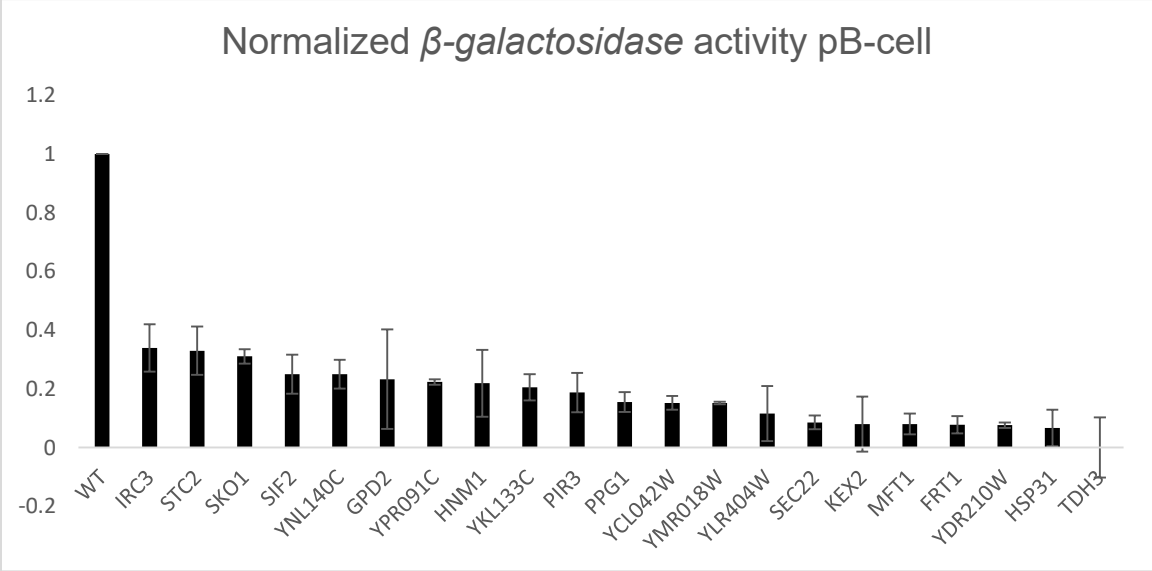
8.2. List of primers used for gene knock out in chapters 2 and 4. Blue and Red color letters are showing complementary regions with NAT sequence in pAG25. Black letters are complementary regions with upstream and downstream of target genes for knock out. Confirmation primers (Conf) are designed for gene replacement confirmation with NAT resistance marker.

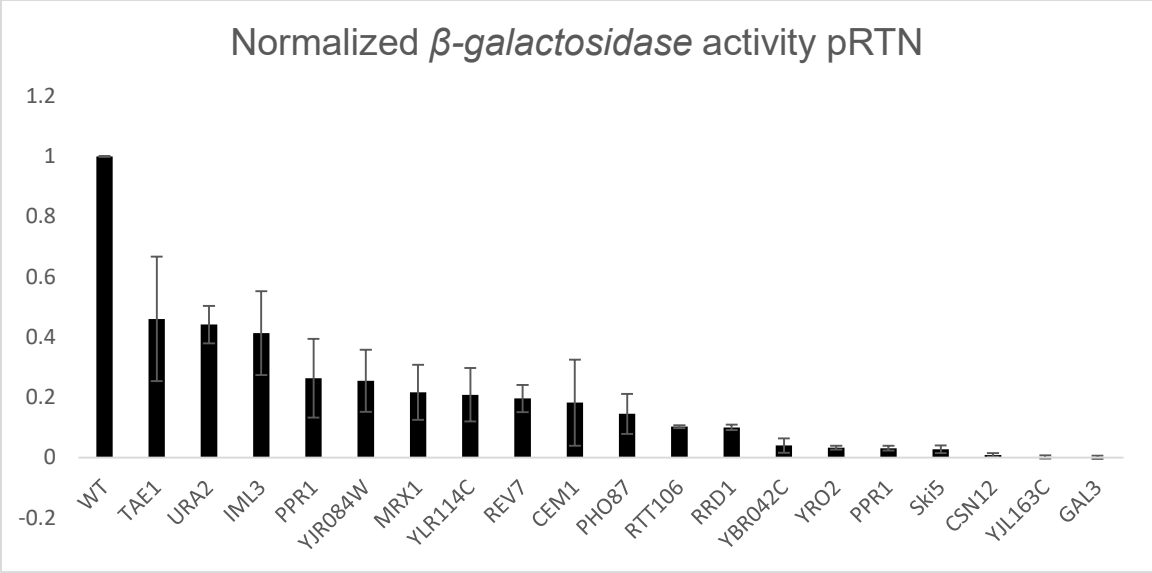
Primer name	Sequence
YTA6-Forward	AGAGGTAAGACCCTGGGTGAAGAAAAGTCCGAGGAGGGAACACAAA AAAGTCTAGGATACACATACGATTTAGGTGACAC
YTA6-Reverse	GTACATAGGTAATAAAATTAGATCAGAAGCAAGAAGTTGAAAGGGG AAAATTCCTTCTCAATACGACTCACTATAGGGAG
YTA6-Conf	AAGGCGGGGCCAAACGAGGCAATC

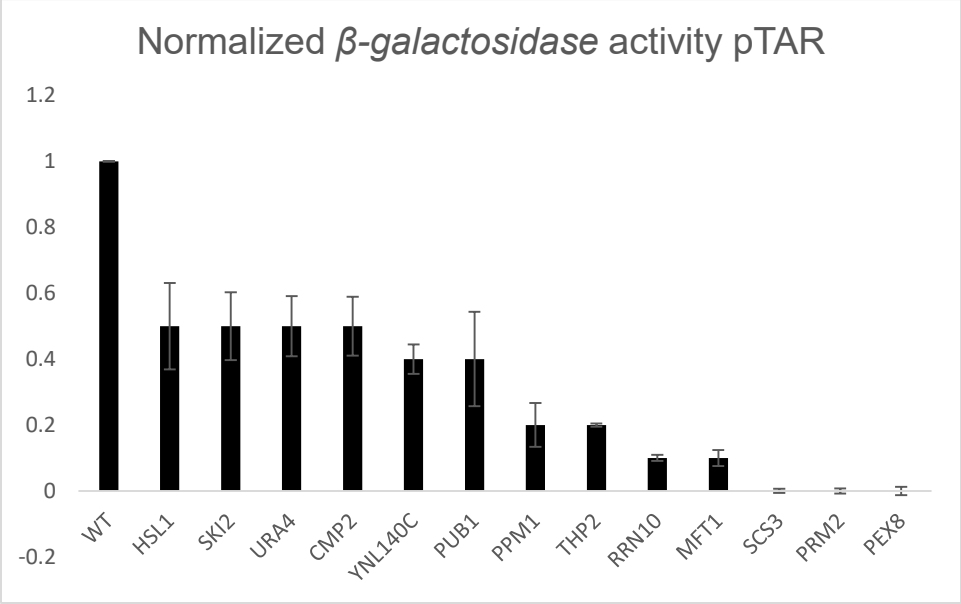
YPR096C-Forward	TTCCTTGTTCACGCCTAAAAAGTTAACTGTCAAATGGTTAACGTACATT TATCTGTCTTCACATACGATTTAGGTGACAC
YPR096C-Reverse	GTTGAAACAGAGTAAATAATAGGGAGTGGATGCGAGTAACCTTGCG ATGTCAAAAGAATAATACGACTCACTATAGGGAG
YPR096C-Conf	GATAGGCTGTGGAGCTGTCGGATCGTTTGG
NAM7-Forward	AGGAAGGGCAGCAAGACCGAATATACTTTTTATATTACATCAATCATT GTCATTATCAACACATACGATTTAGGTGACAC
NAM7-Reverse	TTGAGCCGTTTTGTATCACAAGCCAAGTTTAACATTTTATTTTAACAGG GTTCACCGAAAATACGACTCACTATAGGGAG
NAM7-Conf	TTGCCTGGAGGAAAGGAATCCCAGGAGA
PUS2-Forward	TTACTCCGACACCGTTAACAGCGGTAGTAAAGAGAAGGCCGAGTAAA CATAAGGTCGTTCACATACGATTTAGGTGACAC
PUS2-Reverse	CAACTGGCGCTTGTCTAAGTTGAATAGCCAATAAGATGAGAGTATTG CCCGCCCAATGTAATACGACTCACTATAGGGAG
PUS2-Conf	GGGCCTCCCGCATAAGAACGCACT CC
RPL27B-Forward	TAGTGACTATAGGGCGTTTTGGTGAAAAGAAAGAGTCGCTCAAAGAA ATCAATATAACACATACGATTTAGGTGACAC
RPL27B-Reverse	AAACTGCATTATGAAAATAATAATAGTACACAGAAAAAAGTTAGGTTT CGGAAAAAAGCAATACGACTCACTATAGGGAG
RPL27B-Conf	CTGCCTAGATTGCGCGGAGGGTACGTTG
NAT-Conf	TCCAGTGCCTCGATGGCCTCGGCG

8.3. *β-galactosidase* quantification data for chapter 5. *β-galactosidase* activity of candidate genes, selected from lift assay screening, was performed to confirm the reduction in the translation of *β-galactosidase*. The data was normalized to that of WT carrying either of the constructs p2hair, pB-cell, pRTN, and pTAR.


Normalized β-galactosidase activity p2hair
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
WT
PUS4
FLX1
YMR289W
YNL003C
YNL128W
GAL3
MFT-1
YNL140C
RSC2
SEC22
RTT106
YPL184C
SWI6
NAM7
YNL153C
FRT1
PEX7
TOP1
SET6
THP2
RRN10
URA2
YPL034W


Normalized β-galactosidase activity pB-cell
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
WT
IRC3
STC2
SKO1
SIF2
YNL140C
GPD2
YPR091C
HNM1
YKL133C
PIR3
PPG1
YCL042W
YMR018W
YLR404W
SEC22
KEX2
MFT1
FRT1
YDR210W
HSP31
TDH3


Normalized β-galactosidase activity pRTN
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
WT
TAE1
URA2
IML3
PPR1
YJR084W
MRX1
YLR114C
REV7
CEM1
PHO87
RTT106
RRD1
YBR042C
YRO2
PPR1
Ski5
CSN12
YJL163C
GAL3


Normalized β-galactosidase activity pTAR
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
WT
HSL1
SKI2
URA4
CMP2
YNL140C
PUB1
PPM1
THP2
RRN10
MFT1
SCS3
PRM2
PEX8

8.4. List of candidate genes selected from *β-galactosidase* assay for constructs pTAR, pRTN, pBcell, and p2hair. Function summary of each candidate gens is presented.

Ptar candidate genes	Function summary
RRN10	Regulation in transcription
RPP1A	involved in phosphorylation of eIF2
PEX7	peroxisomal receptor (function as transcription factors regulating gene expression)
PPM1	Carboxyl transferase important in complex formation of regulatory subunits and required for methionine to inhibit autophagy
SCS3	required for inositol prototrophy for regulation of normal ER
THP2 and MFT1	involev in transcription elongation and RNA polymerase II, affecting mRNA export
MET18	component of cytosolic iron-sulfur protein assembly for maturation of some proteins involved in methionine biosynthesis, DNA replication and repair, transcription.
HSL1	protein kinase regulating morphogenesis checkpoint
SKI2	putative RNA helicase. Mediates 3'-5' RNA degradation in exosome important in mRNA decay
URA4	biosynthesis of pyrimidines
CMP2	regulated protein phosphatase involved in stress-response transcription factors
YMR226C	an oxireductaze enzyme, may interact with ribosome
PUB1	poly (A) binding protein, required for stability of many mRNAs. Involved in P-bodies. Translatoi regulation. Protien abundance in response to DNA replication stress
YNL140C	unknown
YOR072W	unknown

pRTN candidate genes	Function summary
SWH1	oxysterol-binding proiten that interacts with ER and is regulated by sterol bindings.
RPL23A	ribosomal subunit
CST26	Acetyltransferase. An enzyme reposible for incorporation of stearic acid into phosphatidylinositol
YRO2	putative role in response to acid stress.
IML3 and SLI15	both important in regulation of kinetochore-microtuble activatoin and mitotic checkpoints
TAE1	methyltransferase, putative role in translatoin
PHO87	phosphate transporter
GAL3	transcriptional regulator

SBH2	involved in protein translocation into ER.
CEM1	a synthase required for mitochondrial respiration.
MRX1	associates with mitochondrial ribosome and its mutation results in a decrease in plasma membrane electron transport.
SAP4	protein required for function of the Sit4 protein phosphate.
SMI1	protein involved in the regulation of cell wall synthesis in coordination with cell cycle progression.
YIL067C	unknown
REV7	subunit of DNA polymerase zeta involved in translesion synthesis in post-replication repair. Involved in double strand break repair. It has domains that are found in proteins involved in cell cycle control, meiosis and DNA reparl.
RRD1	activator of the phosphotyrosyl phosphatase activity of PP2A. Required for rapid reduction of Sgs1p in response to rapamycin. Increases its abundance in DNA replicaton stress.
GUT2	mitochondrial glycerol-3-phosphate dehydrogenase. Invovled in glycerol metabolism and replicative cell aging.
YJL045W	succinate dehydrogenase isozyme involved in cellular respiration, localizes to mitochondria
URA2	carbomylphsphate synthesase involved in biosynthesis of pyrimidines. Because Ura2p is intergral in regulating synhtesis of pyrimidine nucleotides and this process is important in cancer cell metabolism.
YJL163C	unknown
YJR084W	putative role in transcription elongation and mRNA splicing.
PPR1	transciptin factor regulate transcription of genes involved in pyrimidine biosynthesis in response to pyrimidine starvation.
AVL9	involved in exocytic transport from Goli. Abundance upon DNA replication stress.
RTT106	Histone chaperone. Involved in regulation of chromatin structure in both transcribed and silenced chromosmal regions.
SKI3	mediates 3'-5' RNA degradation in exosome important in mRNA decay. Nonsense mediated decay and non stop mRNA decay also mRNAs unadenylated and viral dsRNA.

pBCell candidate genes	**Function summary**
SIF2	subunit of Set3C histone deacetylase complex, repressing early sporulation genes
YCL042W	unknown
PTC1	phosphate involved in inactivation of MAPK activity involved in cell wall intergrity. Pheomone dependent signal transduction and tRNA splicing.
YDR210W	possible plasma membrane related to stress response
IRC3	DNA helicase, deletion cause double stranded breaks in mt DNA.

HSP31	involved in oxidattive stress resistance (defficiency is involved in Parkinson's disease and cancer) contain metal-binding stie. Abundance increases in response to DNA replication and stress.
LSB3	involved in actin filamen assembly. Abundance increases in response to DNA replicatoin stress.
HNM1	plasma membrane transporter active during hypersaline stress localized to ER.
PEX31	Peroxisomal integral membrane protien (a small organelle containing enzyme catalases and oxidases).
TDH3	Glyceraldehyde-3-phosphate dehydrogenase involved in glycolysis, gluconeogenesisi, apoptosis and the matabolism of reactive oxygen species. Binds to AU rich RNAs.
RGT1	DNA-binding transcription factro, acts both as activator and repressor; involved in regulation of glucose metabolism
RCI50	unknown
PIR3	paralog of HSP150. required for cell wall stability. Coding sequence contains length polymorphisms repeats.
SEC22	SNARE protein a group of large protein complex with primary role to mediate vesicle fusion to their target in membrane bound compartments. Deletin cause defective in maturation of cell wall bound proteins and increase sensitivity to cell wall affecting chemicals.
SEI1	protein of the endoplasmic reticulum involved in lipid droplet biogenesis. Stabilizing these sites on ER and deletion causes accumulation of phophatidic acid (PA) marker proteins.
MFT1	involev in transcription elongation and RNA polymerase II, affecting mRNA export
PEX9	Putative signal receptor for peroximsoma matrix proteins; involved in the import of protiens into the peroxisomal matrix. Its conditionn specific receptro.
FET3	integral membrane multicopper oxidase which is required for high-affinity iron uptade and invovled in mediating resistance to copper ion toxicity and abundance increases in response to DNA replication stress.
YNL140C	unknown
SKO1	transcription factor involved in a complex that both activate and repress transcription. Also involved in osmotic and oxidative stress responses. Suppressor of kinase overexpression
KEX2	Kexin a proprotein convertase (these proteins remove block chains on other protiens to activate them) it is calcium dependent and activates proteins on ER (secretory pathway)
PPG1	Putative protein phosphotase, regulates other protien phosphatases.
GPD2	glycerol-3-phosphate dehydrogenase involved in glycerol metabolism and oxidation loicalized in cytoplasm and mt. it is controlled by oxygen-independent signaling pathway required

	to regulate metabolism under anoxic conditions. Inactivated by energy-stress responsice kinase SNF1.
TGL5	triacylglycerol lipase and lysophosphaticid acid (LPA) acyltransferase. Potential substrate of cyclin dependent kinase (CDC28).
WTM1	Transcription factor that regulates meiosis, chromatin silencing inhibits ribonucleotide reductase by sequestering it in the nucleus.
FRT1	an ER membrane protein of unknown function. Substrate of the phospharase calcineurin that promotes cell growth in stress conditions possible via a role in posttranslational translocation.
RPS6A	ribosomal subunit involved in maturaion of the small subunit rRNA and translatoin. Phosphorylated ina TORC1-dependent manner.
NVJ2	lipid binding protein in ER involved in nonvesicular transfer of ceramides form ER to Glogi. May interact with ribosome.
NCA2	protein that regulates expression of Fo-F1 ATP synthase subunits in mt.

p2hair candidate genes	**Function summary**
ERV46	Golgi-vesicle-mediated transport; integral to Golgi and ER membrane.
RRN10	Regulation in transcription
GAL3	transcriptional regulator
MIX14	mt intermembrane space protein of unknown function, protein abundance increases in response to DNA replication stress.
PEX7	peroxisomal receptor (function as transcription factors regulating gene expression)
YDR338C	putative drug transporter
SEM1	Proteasome regulatory component (degrading protiens), involved in mRNA export. Human ortholog DSS1 a BRCA1 binding protein implicated in cancer. Abundance increase in response to DNA replication stress. Deletin exhibits sensitivity o heat and UV
LSM6	cytoplasmic Lsm1p is involved in mRNA decay and possibly involved in processing tRNA, snoRNA and rRNA
SAC7	GTPase activator involved in signal transduction, actin cytoskeleton organization and cell wall organization, potenital substrate of Cycleyn-dependent kinases (cdc28)
SWI4	sequence-specific DNA binding transcription factor during heat stress and the G1/S mitotic transition. Deletion is sensitive to DNA damage, antifungals, alcohols, cold.
PUS2	mt tRNA pseudouridine synthase, mt tRNA modification but mutation also affects pseudouridylatin of some nuclear encoded mRNAs.

THP2	involev in transcription elongation and RNA polymerase II, affecting mRNA export
URA2	carbomylphsphate synthesase involved in biosynthesis of pyrimidines. Because Ura2p is intergral in regulating synhtesis of pyrimidine nucleotides and this process is important in cancer cell metabolism.
SWI6	transcriptin factor involved in meiotic gene expression and regulate transcription at the G1/S transitin. Cell wall stress induce phosphorylatoin of Mpk1p and that regulates Swi6p localizatoi, required for unfolded protein response.
SEC22	SNARE protein a group of large protein complex with primary role to mediate vesicle fusion to their target in membrane bound compartments. Deletin cause defective in maturation of cell wall bound proteins and increase sensitivity to cell wall affecting chemicals.
RSC2	subunit of RSC chromatin remodelong complex, and invovled in transcripton regulatoin for mainraining chromosome transmissino fidelity as well as DNA repair via UV damage excisino, homologous recombination and nonhomologous end joining.
MFT1	involev in transcription elongation and RNA polymerase II, affecting mRNA export
NAM7	ATP-dependent RNA helcase involved in nonsense mediated mRNA decay required for efficient translation termination at nonsense codons and targeting NMD substrates to P-bodies. Binds to Rps26 in ribosome. And forms cytoplasmic foci upon DNA relicatoin stress.
HEF3	Translational elongation factor (EF3). Stimulates EF1 binding of aminoacyl-tRNA by the ribosome.
TEP1	inositol lipid phosphatase activity and homology of human suppressor gene PTEN/MMAC1/TEP1
YNL140C	unkown
RTT106	Histone chaperone. Involved in regulation of chromatin structure in both transcribed and silenced chromosmal regions.
CSE2	subunit of core mediator that acts as RNA polymerase II coactivator required for regulatoin of transcripton by RNA polymerase. Found in cytoplasm under hypoxia. In mutation cell is sensitive to chemicals including caffeine, rapamaycin, hygromycin B.
TOP1	DNA topoisomerase the unwinds DNA, chromatin organizition, DNA replication and transcription and in replication checkpoint signalng. Target of antitumor drug camptothecin. Drug increases the half-life enzyme DNA complex which results in increase of double stranded DNA breaks during DNA replicatoin.

CIN5	transcription factor, mediates pleiotropic drug resistance and salt tolerance, activated during oxidative stress. Overexpression elevates resistance to mitomycin C and cisplatin
FRT1	an ER membrane protein of unknown function. Substrate of the phospharase calcineurin that promotes cell growth in stress conditions possible via a role in posttranslational translocation.
SKS1	putative serine/threonine protein kinase, suppressor kinase SNF3 (plasma membrane). Invovled in adaptaion to low concentratio of glucose
YPL034W	unkown
SET6	SET domain of unknown function
MRN1	RNA-binding protein that may be involved in translatoinal regulation; binds to specific categories of mRNAs, including those that contain uORFs and IRES. Genetically interacts with chromatin remodeling and splicing factors and mRNA maturation.

8.5 Selected manuscripts

1. Burnside, D., Schoenrock, A., Moteshareie, H., Hooshyar M., Basra, P., **Hajikarimlou, M**., Dick, K., Barnes, B., Kazmirchuk, T., Jessulat, M., Pitre, S., Samanfar, B., Babu, M., Green., J., Wong, A., Dehne, F., Biggar, K and Golshani, A. (2019). **In Silico Engineering of Synthetic Binding Proteins from Random Amino Acid Sequences in Silico Engineering of Synthetic Binding Proteins from Random Amino Acid Sequences.** ISCIENCE, 11, 375–387. https://doi.org/10.1016/j.isci.2018.11.038.

2. Moteshareie, H., **Hajikarimlou, M.**, Indrayanti, A. M., Burnside, D., Paula, A., Letti, C., Omidi, K., Kazmirchuk, T., Puchacz, N., Zare, N., Takallou, S., Naing., T., Hernandez, R.B., Willmore, W.G., Babu, M., McKay, B., Samanfar, B., Holcik, M and Golshani, A. (2018). **Heavy metal sensitivities of gene deletion strains for ITT1 and RPS1A connect their activities to the expression of URE2, a key gene involved in metal detoxification in yeast. Plose One.** 1–18. https://doi.org/10.1371/journal.pone.0198704.

3. Omidi, K., Jessulat, M., Hooshyar, M., Burnside, D., Schoenrock, A., Kazmirchuk, T., **Hajikarimlou, M**., Daniel, M., Moteshareiri, H., Bhojoo, U., Sanders, M., Ramotar, D., Dehne, F., Samanfar, B., Babu, M and Golshani, A. (2018). **Uncharacterized ORF HUR1 in fl uences the e ffi ciency of non-homologous end-joining repair in *Saccharomyces cerevisiae***. Gene. 128–136. https://doi.org/10.1016/j.gene.2017.10.003.

4. Kazmirchuk, T., Dick, K., Burnside, D., Barnes, B., Moteshareie, H., **Hajikarimlou, M**., Omidi, K., Ahmed., Duale., Low, A., Letti, C., Hooshyar, M., Schoenrock, A., Pitre, S., Babu, M., Cassol., E., Samanfar, B., Wong A., Dehne, F., Green J.R and Golshani A. (2017). **Designing Anti-Zika Virus Peptides Derived from Predicted Human-Zika Virus Protein-Protein Interactions.** Computational biology and chemistry. 71, 180-187. https://doi.org/10.1016/j.compbiolchem.2017.10.011

5. Samanfar, B., Shostak, K., Moteshareie, H**., Hajikarimlou, M**., Shaikho, S., Omidi, K., Hooshyar, M., Burnside, D., Marquez, I.G., Kazmirchuk, T., Naign, T., Ludovico, P., York-lyon, A., Szereszewski, K., Leung, C., Jin, J.Y., Megarbance, R., Smith, M.L., Babu, M., Holcik, M and Golshani, A. (2017). **The sensitivity of the yeast, Saccharomyces cerevisiae, to acetic acid is influenced by DOM34 and RPL36A.** PeerJ, 2017(11). https://doi.org/10.7717/peerj.4037.

6. Shaikho, S., Dobson, C. C., Naing, T., Samanfar, B., **Hajikarimloo, M**., Golshani, A., & Holcik, M. (2016). **Elevated levels of ribosomal proteins eL36 and eL42 control expression of Hsp90 in rhabdomyosarcoma.** Translation, 4(2), 1–12. https://doi.org/10.1080/21690731.2016.1244395.

7. Barnes, B., **Hajikarimlou, M**., Schoenrock, A., Burnside, D., Cassol, E., Wong, A., Dehne, F., Golshani, A and Green, J.R. (2016.). **Predicting Novel Protein-Protein Interactions Between the HIV-1 Virus and Homo Sapiens.** 2016 IEEE EMBS International Student Conference (ISC), 1, 1–4. https://doi.org/10.1109/EMBSISC.2016.7508598.