Investigation of heavy metal toxicity leads to the identification of
novel translation associated genes in yeast (*Saccharomyces
cerevisiae*)

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Abstract

Heavy metal and metalloid pollutants in our environment are among the most concerning types of contaminations. Major chronic diseases in humans such as renal and cardiovascular diseases, and neurological decline, are strongly associated with heavy metals and metalloids. Therefore, investigation and understanding the molecular mechanisms of cellular responses and detoxification processes that overcome the toxicity of these compounds in living organisms is very important. To date, several genes are identified to play central roles in cellular detoxification process. The expression of such genes can be influenced at both the transcriptional and/or translational levels by the heavy metals. As a fundamental step in the gene expression pathway, we focused on the regulation of translation initiation under stress imposed by heavy metals and metalloids. Although a wealth of information exists on the process of eukaryotic translation, a comprehensive understanding of regulation of translation initiation under stress conditions is lacking. The growing list of novel factors affecting this process further indicate the existence of other novel players, which are yet to be discovered. In the current study, we sought out to identify novel genes encoding regulatory factors known that affect yeast translation initiation during stress when general translation seems to be shut down. Utilizing systems biology techniques, we investigated the effect of specific gene deletions under heavy metal and metalloid conditions on the general process of translation and internal initiation of translation (an alternative mode of translation mediated by specific RNA structures). We explicitly investigated the role of four of identified potential translation regulating proteins based on their activity in heavy metals and metalloids sensitivity. We also performed a high-throughput plasmid-based screening of a library of non-essential gene deletion strains (~4500), using the baker's yeast (*Saccharomyces cerevisiae*) as our model organism to identify novel genes that are involved in internal translation initiation. To this end, we identified dozens of potential novel genes that may be involved in internal translation initiation. We further investigated the role of five potential factors to support their newly identified activity.

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Statement of contribution

The thesis, “Investigation of metal toxicity leads to the identification of novel translation associated genes in yeast (*Saccharomyces cerevisiae*)” is composed of four study projects.

Chapter 2, “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”, 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 co-supervision. Ana Paula Dias helped me with SGA and PSA analysis, Clara Lettl helped me with western blot analysis, Alex Mulet Indrayanti helped me with polysome profile analysis and other experiments. I personally wrote the first draft of the manuscript.

Chapter 3, “Deleting *UGA4* and *YBR062C* increases heavy metal sensitivity in yeast, *Saccharomyces cerevisiae*”, 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 co-supervision. Ana Paula Dias helped me with SGA and PSA analysis, Clara Lettl helped me with western blot analysis, Alex Mulet Indrayanti helped me with polysome profile analysis and other experiments. I personally wrote the first draft of the manuscript.

Chapter 4, “4 Chapter 4: Large-scale investigation of multiple IRES elements in yeast, *Saccharomyces cerevisiae*”, is the result of experiments primarily designed by me and carried out by me and Alex Mulet Indrayanti (undergraduate student). This chapter was partially performed as an honours project by Alex Mulet Indrayanti.

Chapter 5, ‘Manganese-induced cellular disturbance in the baker’s yeast, *Saccharomyces cerevisiae* with putative implications for neuronal dysfunction provoked by this metal’’, is a collaborative effort between Dr. Hernandez and our lab. This project was designed by Dr. Hernandez and Dr. Golshani and the experiments were performed by me and Dr. Hernandez. I performed polysome profiling and assisted him in all other experiments and analysis. This manuscript was written by Dr. Hernandez and me together. Daniel Burnside assisted this project experimentally and analytically.

During the completion of my PhD studies at Carleton University, I have collaborated in a diverse range of interesting projects majority of which have been published or submitted to a journal for peer reviewed publication. The list of my current peer-reviewed publications is presented in Appendix 7-1.

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List of abbreviations

µg	Micro grams
µL	Micro liters
°C	Degrees Celsius
AD	Activation domain
ATP	Adenosine triphosphate
cDNA	Complementary DNA
clonNAT	Nourseothricin
DB DNA	Binding Domain
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
eEFE	eukaryotic elongation factor
eIF	Eukaryotic initiation factor
g	Gram
G418	Geneticin
GAL	Galactose
GD	Growth detector
GI	Genetic interaction
IRES	Internal ribosome entry site
ITAFs	IRES transacting factors
LB	Lysogeny broth
YPD	Yeast Peptone D-glucose
YNB	Yeast Nitrogen Based
MAT	Mating type locus
mg	Milligram
min	Minute
mL	Milli liter
mM	Milli molar
mRNA	Messenger RNA
tRNA	Transfer RNA
rRNA	Ribosomal RNA

ng	Nanogram
OD	Optical density
ONPG	O-nitro-phenyl- β -D-galactoside
ORF	Open reading frame
PABP	Poly A binding protein
PCD	Programmed cell death
PCR	Polymerase chain reaction
PPI	Protein-protein interaction
PSA	Phenotypic suppression analysis
RT-qPCR	Reverse transcriptase-quantitative PCR
rDNA	Ribosomal DNA
RF	Release factor
RNA	Ribonucleic acid
RT	Room temperature
SC	Synthetic complete media
SDL	Synthetic dosage lethality
SDS-PAGE	Sodium dodecyl sulfate poly-acrylamide gel electrophoresis
SGA	Synthetic genetic array
SGD	Saccharomyces genome database
TAE	Translation associated element
TC	Ternary Complex
UTR	Untranslated region
URA	Uracil
UV	Ultraviolet
WT	Wild type
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
YPD	Yeast extracts peptone dextrose media
As	Arsenic
Mn	Manganese
Ni	Nickel
Cd	Cadmium

H₂O	Water
YKO	Yeast MATa KnockOut
GABA	4-aminobutyric acid

1 Chapter 1: Introduction

1.1 Heavy metals and their toxicity

Heavy metals are ubiquitous naturally occurring elements in the environment that have a high atomic weight and possess a density of more than 5 g/cm³ (i.e. at least five times greater than water) (Jaishankar *et al.*, 2014). Several heavy metals including iron (Fe), copper (Cu), cobalt (Co), zinc (Zn), magnesium (Mg) and manganese (Mn) function as essential elements to maintain various biochemical and physiological functions in living organisms and have the capability of mediating several cellular processes via interactions with protein and DNA/RNA (Jaishankar *et al.*, 2014). Other heavy metals including rubidium (Ru), silver (Ag) and indium (In) are relatively harmless at a low concentration. However, according to the US Environmental Protection Agency and the International Agency for Research on Cancer, almost all heavy metals and metalloids have the ability to induce various diseases above the physiologically required levels and are carcinogens at high concentrations (Lin, 1998; Godt *et al.*, 2006; Violante *et al.*, 2010; Tchounwou *et al.*, 2012). The cellular and molecular pathways that govern the toxicity of heavy metals and related diseases in living organisms have been the subject of vigorous investigations (e.g. Lin, 1998; Hall, 2002; Godt *et al.*, 2006; Violante *et al.*, 2010; Moteshareie *et al.*, 2018).

Heavy elements such as heavy metals are found in wide range of concentrations deposited across the earth's crust in our environment. As mentioned above, some are essential elements while others are relatively harmless at low concentrations, including Ru, Ag, and In. A few other heavy metals including cadmium (Cd), Nickel (Ni), mercury

(Hg), arsenic (As) and chromium (Cr) are highly toxic to all living organisms, even at a very low concentration (Tchounwou *et al.*, 2012). The presence of toxic metals in our environment is a result of two main events, they are either derived from natural activates (e.g. weathering and volcanic activities) or anthropogenic sources such as industrial, domestic, agricultural, medical, and technological products (e.g. phosphate fertilizers, disinfectants, fungicides, sewage sludge, industrial waste, and dust from smelters) (Schwartz *et al.*, 2001; Passariello *et al.*, 2002). This wide distribution of toxic metals in the environment has raised concerns over their potential effects on human health and other living organisms in the environment.

The toxicity of heavy metals and metalloids in humans generally depends on multiple factors including the type of metal, route of exposure, chemical species, dose, as well as the genetic, gender, nutritional status, and age of the exposed person. Toxic metals including Cd, As, Ni, Cr, Hg and Pb hold high toxicity and their exposure is ranked among the priority metals that significantly concern the public health (Tchounwou *et al.*, 2012). These heavy elements are known to cause systemic toxicities that are the major cause of multiple organ damage and failure, even at very low contact levels (Jaishankar *et al.*, 2014; Rana and Rahman, 2018).

Mammalian organisms are primarily exposed to heavy metals via consumption of polluted food and water contaminated from mining, plastics and disposed batteries (Rana and Rahman, 2018). In these organisms, As, Cd, Pb, and Hg do not play vital biochemical roles, but they impose several severe toxicities in multiple organs as they can bind to different tissues at the cellular level, creating oxidative stress that leads to endocrine disfunction, aquaporins blockage, and disrupt the functions of essential cation ions

including Zn, Mg and Mn (Sears, 2013). In mammals, toxic metals are excreted through kidneys which makes them the most vulnerable organ to heavy metal toxicity (Monnet-Tschudi *et al.*, 2006; Rana and Rahman, 2018). Other organs including lungs and brain are also fatally affected by even a low concentration of heavy metals and metalloids present in blood composition (Monnet-Tschudi *et al.*, 2006). In addition, major chronic diseases in adult individuals such as renal and cardiovascular diseases, and neurological decline, are also strongly associated with exposure to heavy metals and metalloids (Alwan, 2011).

1.1.1 Cellular response to heavy metal toxicity

The mobility of toxic metals, their excretion and accumulation in each organ is linked to their oxidation state, solubility and active transport via membranes (Apostoli, 2006; Ayangbenro and Babalola, 2017). The strength of a toxic damage depends on the organism, cell or tissue type, absorption, concentration, environment, location of action and type of metal (Tchounwou *et al.*, 2012). Untreated free toxic metals with oxidation states greater than one (i.e., a charge of +2 or more) are redistributed throughout the body and reimpose toxicity at intracellular sites within the kidney and liver (Flora *et al.*, 2010). Different antioxidant treatments, including nutrient and/or vitamin administrations, can reduce the toxicity imposed by toxic metals. These compounds act as chelating agents to reduce toxic metals to a lower redox state which is eventually excreted via blood circulation through kidneys (Hsu and Guo, 2002; Flora *et al.*, 2010). The basic mechanisms that facilitate resistance to these heavy elements and their detoxification take place at the cellular levels via several complex biochemical pathways. These pathways are evolved to survive acute oxidative stress from high concentrations of heavy metals

due to the prolonged exposure in their environment (e.g. Basu *et al.*, 1997; Choudhury and Kumar, 1998; Castro-Silva *et al.*, 2003; Otth *et al.*, 2005). Plants are exposed to oxidative stress, created by heavy metals through agricultural products and polluted environments which leads to cellular damage (Basu *et al.*, 1997; Castro-Silva *et al.*, 2003). They have evolved detoxifying biochemical pathways at the cellular levels to minimize the detrimental effects of heavy metals. These detoxification mechanisms are primarily dependent on chelation and subcellular accumulation and compartmentalization. The rate of tolerance to heavy metals and metalloids in plants, can be associated to their ability to live in a polluted environment in comparison to other plants and is demonstrated by genotypic interactions with the environment (Macnair *et al.*, 2000). This can be also regulated indirectly by general chemical responses associated with redox transitional organic molecules such as sugar, nucleic acids, amino acids, organic acids, glutathione and vitamins.

In this context, microorganisms that can tolerate the toxicity of heavy metals also indirectly contribute to the reduction of oxidation state in the soil. In several studies, tolerance to heavy metals has been suggested to be governed by a few major genes or gene families including *PCS* gene family in fungi and *URE2* in yeast. It is thought that these major genes are perhaps regulated by a number of minor regulator genes (Macnair, 1993; Clemens *et al.*, 1999; Macnair *et al.*, 2000; Schat *et al.*, 2000; Rai *et al.*, 2003). Detoxification by affiliated microbiome contributes to the complexity of the biochemical and molecular pathways required to produce tolerance in higher organisms. This urges us to investigate and understand the intermediate genes involved in the process of detoxification in all organisms including microorganisms.

Single celled-organisms have been exposed to toxic elements in the environment for more than 3.5 billion years (Schopf *et al.*, 2007). Natural populations of microbes in polluted areas are subjected to a selective process for an increased resistance to toxic metals during the course of evolution (Gadd, 2005). Many microorganisms in water, soil and industrial waste have developed resistance to heavy metals by using detoxification pathways to reduce their oxidative state to a lower redox state, which plays a key role in the mobilization and immobilization of metal cations (Gadd, 2005). Several genes such as Phytochelatin Synthases (PCS) gene family (Clemens *et al.*, 19990) encode proteins that facilitate resistance to a variety of metal ions. In addition, many microorganisms use metals, including Co, Cu, Ni and Mn, as micronutrients for redox processes, to regulate osmotic pressure, stabilize molecules through electrostatic interactions, and as components of various enzymes (Bruins *et al.*, 2000). As an example of well-adopted microorganisms to toxic metals, the baker's yeast, *Saccharomyces cerevisiae* benefits from an effective mechanism to negate the toxicity levied on the yeast cells by heavy metals, allowing them to survive a broad range of toxic stress conditions (Vadkertiová and Sláviková, 2006; Hosiner *et al.*, 2014). In addition to its strong mechanism against toxic metals, this organism is a very well-studied and adopted to laboratory conditions, its genome was the first eukaryotic genome to be sequenced and different library collections (e.g. deletion, over-expression and GFP infused collections) were created that make yeast a formidable model organism to study molecular mechanisms of the stress response that drive detoxification processes.

1.1.2 Effect of toxic metals and their mode of action at the cellular levels

Heavy metals and metalloids can interact with living cells in various ways, they

commonly have diverse chemical and physical properties and hence their toxicity can be exerted in several ways including dislocation or substitution of essential metal ions from biomolecules, conformational modifications, denaturation and disfunction of enzymes, disruption in the integrity of cellular and organelle membrane and blocking the functional groups (Gadd, 1993; Soares *et al.*, 2003; Ayangbenro and Babalola, 2017).

One of the primary effects of toxic metals at the cellular level is damage to the cell membrane, particularly the plasma membrane. More specifically, a complex interaction between heavy metals and the functional groups of membrane components can lead to disruption of membrane integrity (Janicka-Russak, 2008; Ayangbenro and Babalola, 2017). Metal ions effortlessly bind to proteins at the sulfhydryl groups and to phospholipids at the hydroxyl region. In these interactions, heavy metals can be easily substituted and/or replaced by calcium ions at essential sites on the cell membranes (Devi and Prasad, 1999; Janicka-Russak, 2008). These cellular chemical reactions enforced by the presence of toxic heavy elements, can directly increase non-specific membrane permeability. In parallel, these interactions can decrease corresponding transporting chemical events that maintain the ionic homeostasis that can eventually hinder the activities of many enzymes that play critical roles in basic cellular metabolism (Soares *et al.*, 2003; Janicka-Russak, 2008; Ayangbenro and Babalola, 2017).

The effect of heavy metal and metalloids is not limited to the cell membrane, and they can affect other cellular processes as well. Generally, these heavy elements can react with endogenous essential biomolecular targets such as proteins, receptors, enzymes, lipids, RNA and/or DNA molecules. These interactions can critically alter the dynamics

and homeostasis of the cell, producing functional and structural fluctuations that can cause severe damage that in certain cases may lead to apoptosis (Shanker, 2008). Once an intracellular target, playing a regulatory, signaling and/or maintenance role is challenged by heavy metal elements, it can trigger a cascade of effects including improper cell division and apoptosis due to changes in gene expression, impaired ATP synthesis, changes in internal and external charges, cell extracellular and impaired protein synthesis (Prasad, 2008; Ayangbenro and Babalola, 2017). Different metals may pose specific mode of action, by understanding the mechanisms of toxicity for individual toxic metal, we can better understand the general and specific mechanisms of resistance and detoxification pathways. The current study focuses on protein synthesis and the effect of heavy metals on this essential process.

The response of living organisms to toxicity of a heavy metal may often lead to an observable phenotype. For example, fermentation in yeast can be partially repressed as a result of exposure to 2 mM Ni (Ross, 1975). The same concentration of Ni can also inhibit glucose uptake (Jones and Greenfield 1984); and/or inhibit synthesis of RNA and proteins (Joho *et al.*, 1987). The presence of 1 μ M Cu or above in the cells can inhibits glycolytic flux, above 10 μ M Cu can inhibit growth, and at 0.8 mM and above can induce apoptosis in yeast cells (Jones and Greenfield, 1984). Exposure to Cu can also have morphological effects and a drop in intracellular pH level (Ohsumi *et al.*, 1988). Cd and As are none-essential heavy metal and metalloid that even at very low concentrations can have strong effects on microbial metabolisms (Gadd, 1993; Ross, 1975; Rai *et al.*, 2003; Rai and Cooper, 2005; Moteshareie *et al.*, 2018).

Due to the negative charge of DNA when a toxic metal is transported inside the nucleus, it can often bind to DNA strands (Jackson and Bartek, 2009). Duguid *et al.*, in 1993, suggested that ion partners from heavy metals can induce irreversible conformational damage on DNA double helix *in vitro*. The interactions between metal ions and DNA may disturb the functional relationships between DNA molecules and DNA binding proteins (Sas-Nowosielska and Pawlas, 2015). In the same context, gene expression can be inhibited when the large grooves of DNA are twisted around the Cr(III) molecules on opposite sides, which blocks transcription factors from interacting with the double helix (Raja and Nair, 2008).

In most microorganisms, including yeast, metal intake first initiates with a nonspecific binding of the metal to the cell membrane. As explained above, this interaction can trigger a cascade of biochemical reactions that leads to functional changes in cellular membrane (Prasad, 2008; Ayangbenro and Babalola, 2017). These metals, after interaction with the cell membrane, can generally travel further across the membrane by a slower intracellular metabolism-dependent intake process. The secondary interactions across the membrane, inside cytoplasm, holds a major impact on the cellular metabolism and consequently causes the activation of different intracellular biochemical pathways. These pathways eventually either can trigger defense mechanisms or stimulates apoptosis (Blackwell *et al.*, 1995; Ayangbenro and Babalola, 2017).

The last course of action that is triggered by heavy metal toxicity is the repair mechanism (Shanker, 2008; Park and Jeong, 2018). Heavy metals can damage DNA, cell membrane, and even tissues or organs (Flora *et al.*, 2010). In the presence of toxic metals, any damage to the repair mechanisms can have irreversible effect at higher levels of the

biological hierarchy of a cell. The resistance to toxic metals via repair pathways can take place at cellular, molecular, and tissue levels (Shanker, 2008; Park and Jeong, 2018).

1.1.2.1 Mechanism of resistance

Living organisms have developed specific genetic abilities to survive high concentrations of toxic heavy metals (Gupta and Diwan, 2017). It is thought that the diverse adaptation and resistance mechanisms to heavy metals have been selected in a similar way that antibiotic resistant strains are selected in the environment (McIntosh *et al.*, 2008; Gupta and Diwan, 2017). An association between heavy metal resistance and antimicrobial resistance is also supported by the location of both genes frequently co-occurring on the same mobile genetic elements. (McIntosh *et al.*, 2008; Lima e Silva *et al.*, 2012; Kiran *et al.*, 2017). The mechanism of resistance to toxic metals is generally proposed to be divided into five categories: 1) permeability barrier and exclusion of the metal, 2) elimination by active transfer of the metal to outer membrane, 3) intracellular physical isolation of the toxic metal by binding to proteins or other ligands to avoid harmful effect on the metal sensitive intercellular targets, 4) extracellular sequestration (chelation and cell-wall binding, and 5) transformation via detoxification. The resistance of microorganisms to heavy metals not only depends on the molecular pathways that mediate detoxification, it is also influenced by several conditional factors such as concentration of chelating agents, speciation, pH, and organic matter. The arrangement of these elements by the environment determines the biodiversity of microbial communities and cycling of element (Lima e Silva *et al.*, 2012). In the past studies, fungi resistance mechanisms to heavy metal ions has been examined and enhanced to increase the tolerance of fungi for the industrial bioleaching or microbial ore leaching process where

microorganisms are used to extract metals from their ores (Choudhury and Kumar, 1998).

At the molecular level, the glutathione S-transferases (GSTs) are key enzymes that mediate the resistance of various organisms to a wide range of heavy metals and metalloids. Yeast *Ureidosuccinate Transport 2 (URE2)* gene product is structurally homologous to mammalian GST and is a major player in the detoxification of *S. cerevisiae* against toxic metals through its glutathione peroxidase activity (Umland *et al.*, 2001; Bai *et al.*, 2004). For detoxification purposes, GST proteins catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates (Todorova *et al.*, 2010). The deletion strain for *URE2* is hypersensitive to a wide range of heavy metals and metalloids including As, Cd and nickel (Ni) Todorova *et al.*, 2010; Rai *et al.*, 2003). The expression of *URE2* at the protein levels is one of the main focuses of my thesis. Other genes that influence heavy metal and metalloid toxicity in *S. cerevisiae* includes ARsenicals Resistance 3 (*ARR3*), Thiol-Specific Antioxidant (*TSAl*) and Bile Pigment Transporter (*BPT1*) (Chae *et al.*, 1993; Petrovic *et al.*, 2000; Meng *et al.*, 2004; Markowska *et al.*, 2015; Irokawa *et al.*, 2016). Toxic metals may trigger very specific pathways in living organisms. For example, As resistance machineries in majority of microorganisms is mediated by four different types of transmembrane channels or transporters (Ghosh *et al.*, 1999; Meng *et al.*, 2004). In *Escherichia coli* arsenite efflux is controlled by ArsB (Meng *et al.*, 2004), in *Sinorhizobium meliloti* arsenite is exported to the outer membrane via the Major Intrinsic Protein AqpSp (Yang *et al.*, 2005) and in yeast, *S. cerevisiae* detoxification of arsenite is achieved by arsenite efflux either through Arr3p or through compartmentalization of glutathione-arsenic adducts into the vacuole by Yeast Cadmium Factor Protein (Ycf1p) or excretes into the outer membrane via

glutathione S-transferases (GSTs) proteins such as Ure2p (Ghosh *et al.*, 1999; Bousset *et al.*, 2001; Markowska *et al.*, 2015).

Cadmium as an example of a carcinogenic heavy metal, is a cumulative toxin and different rates of its absorption can exert varying adverse health effects. The concentration of Cd in mammalian systems increases over time due to its slow detoxification process. Cd is primarily accumulated in the liver and kidneys and minorly in muscles and bones. However, the kidneys, bone, and lungs are severely affected by cadmium's toxicity, both acutely and chronically. The lungs can be damaged by exposures to acute inhalations in addition to suffering from more chronic exposures as a result of occupational hazards. The kidneys can be damaged more commonly as long-term chronic exposures however acute high-dose can also be harmed. The bone disease resulted from exposure to high dose of Cd is known to be secondary to cadmium's effects on the kidneys (Sahmoun *et al.*, 2005).

Cd is a known catalyst in the formation of ROS and is known to increase oxidative stress by increasing the lipid peroxidation, and depleting glutathione and protein-bound sulfhydryl groups. Cd toxicity is also known to downregulate the protective activity of nitric oxide formation that stimulates the production of inflammatory cytokines (Navas-Acien *et al.* 2004). This heavy metal also exerts its toxic effect by regulating the genotoxic activities both *in-vitro* and *in-vivo* however there is limited epidemiological evidence for *in vivo* human genotoxicity. Cd has been found to induce chromosomal damage in cadmium-exposed workers by showing multiple chromosomal aberrations in the lymphocytes. This carcinogenic heavy metal is also known to cause cell

transformation, DNA strand breaks impaired DNA repair, multiple form of mutations, impaired signal transduction and gene expression (Waisberg *et al.*, 2003).

Arsenic (As) is a highly toxic metalloid and daily exposure of living organisms is due to environmental pervasiveness. As is found in several different chemical forms and oxidation states, which can cause acute and chronic health issues, including cancer. The metabolism of arsenic has an important role in its toxicity. The exact mechanisms that As exerts its toxicity is not well known, but several hypotheses have been proposed. The metabolism that processes As toxicity involves reduction to a trivalent state and oxidative methylation to a pentavalent state (Katsoyiannis *et al.*, 2015). The trivalent arsenicals, including those methylated, have more potent toxic properties than the pentavalent arsenical (Dahlawi *et al.*, 2018). In the pentavalent state, inorganic As can replace phosphate in several reactions. At this state, both organic and inorganic methylated As may react with critical thiols in proteins which can inhibit their function (Katsoyiannis *et al.*, 2015). The potential mechanism proposed in cancer patients include altered DNA methylation, genotoxicity, co-carcinogenesis, altered cell proliferation and oxidative stress which can all induce tumor promotion (Steinmaus *et al.*, 2013). A better understanding of the mechanism(s) that govern arsenic toxicity will make a more confident determination of the risks associated in living organisms with exposure to this toxic chemical.

1.1.2.2 Heavy metals can influence gene expression

Several studies have shown that metal-regulated gene expression plays imperative role in the metabolism of heavy metals and cellular protection from their toxicity (e.g.

Korashy *et al.*, 2005; Kiran *et al.*, 2017; Moteshareie *et al.*, 2018). Depending on the chemical properties of the individual metal each heavy metal has specific effects on biological systems (Butt *et al.*, 1985; Korashy *et al.*, 2005; Tamás *et al.*, 2014). Various cellular interactions that are generated in the presence of different heavy metals generally lead to the activation of detoxification pathways (Rai *et al.*, 2003; Yadav, 2010; Moteshareie *et al.*, 2018). Several heavy metals may activate similar detoxifying pathways (Yadav, 2010; Moteshareie *et al.*, 2018). For example, the resistance of *S. cerevisiae* to a wide range of heavy ions such as As, Cd and Ni is synchronized by triggering glutathione peroxidase activity and glutathione S-transferase products (Rai *et al.*, 2003; Todorova *et al.*, 2010). Presence of toxins such as that imposed by toxic metals can change the expression profile inside the cells. This can in return change the expression levels of several genes involve in detoxification. For example, the presence of arsenite can upregulate the expression of *ARSB* gene in *E. coli*, *AQPS* gene in *S. meliloti*, and *ARR3* gene in *S. cerevisiae* (Ghosh *et al.*, 1999; Meng *et al.*, 2004; Yang *et al.*, 2005).

It is also suggested that the toxic metals can cause stress on endoplasmic reticulum (ER) and eventually lead to apoptosis. These types of stresses and the countereffects can cause suppression or inhibition of canonical cap-dependent translation (Johnson *et al.*, 2017). During these stress conditions, the expression of certain genes must be actively carried out and tuned to battle the toxicity effect of heavy metals for cell survival. The cell targets protein synthesis as the fastest way to alter the expression of such critical genes. However, when canonical translation is inhibited, these essential genes for stress survival must be constantly expressed hence the cell must have developed other ways of translation to overcome the inhibition effects caused by these conditions.

1.2 Protein synthesis

It is generally thought that the expressions of eukaryotic genes are controlled at two separate processes both of which are part of the central dogma of biology. The first process is transcription by which DNA is encoded into a messenger RNA (mRNA). Following this process, eukaryotic mRNAs undergo a series of complex biological processes and dynamic structural rearrangements before it is used as a template for protein synthesis by which the genetic information on mRNA molecules are converted into proteins. The basic process of protein synthesis is well conserved across all living organisms, yet the molecular details can differ among species for any of the major phases of the translation cycle. This biological cycle begins with the assembly of ribosomal machinery, containing a transfer RNA (tRNA) molecule, specialized for initiation (Met-tRNAⁱ) carrying initiator methionine amino acid (Fig 1-1) (Kapp and Lorsch, 2004; Schuller and Green, 2018).

Ribosomes are the molecules responsible for protein synthesis by decoding triplet codons carried by an mRNA molecule. Ribosomes are complex molecules that structurally are made up of ~ 60% ribosomal RNAs (rRNAs) and ~40% protein. Eukaryotic ribosomes have two major subunits; one large (60S) and one small (40S). The smaller subunit of the ribosome binds to the 5' end of the mRNA, while the larger subunit binds to the tRNA. The two subunits unite near the initiation codon to form a complete ribosome (80S) capable of mediating the synthesis of polypeptide chains. When a cycle of translation process is completed, the complex of mRNA and 80S ribosome will either disassemble or reinitiate the translation of the same mRNA, forming a loop (Wilson and Cate, 2012).

In a classical view of translation, this process is often divided into three major phases: initiation, elongation and termination, which together produce a translation product (Fig 1-1). Due to its complexity, most regulation of translation is exerted at initiation step, where the start codon (AUG) is identified and translated by the Met-tRNA_i (Sonenberg and Hinnebusch, 2009). Translation and regulation of translation (translation control) have been investigated for several decades (Kapp and Lorsch, 2004; Sonenberg and Hinnebusch, 2009; Wilson and Cate, 2012; Schuller and Green, 2018). Although much has been learned, details of factors that influence the efficiency of this process as well as its control and communication with other cellular processes are not fully understood yet. This study aims to identify new factors that can influence the translation process under normal and physiological stress conditions.

Figure 1-1: General schematic of eukaryotic translation process underlying the three main steps of eukaryotic protein synthesis. It is believed that translation starts by the binding of 40S ribosomal subunit to mRNA. This is followed by the recruitment of 60S ribosomal subunit to the complex, initiating the synthesis of the polypeptides. During termination a stop codon is encountered by the translation machinery which releases the polypeptide chains.

1.2.1 Initiation of translation

It is generally accepted that the majority of regulation of translation takes place during the initiation phase. Consequently, initiation has been the subject of vigorous study

to explain the molecular details of any potential control elements and pathways. Eukaryotic initiation of translation can be subdivided into two mechanisms: cap-dependent and cap-independent pathways. In the cap-dependent mechanism, initiation of translation consists of a set of intensive reactions that recruit ribosomal subunits along with associated proteins and tRNAs to the mRNA and shift the machinery to place an appropriate AUG start codon from the mRNA in the P-site (Peptidyl-site) of the ribosome. The translation machinery is then base-paired with the anticodon of Met-tRNA_i through the scanning mechanism (Fig 1-2). This whole process starts with the assembly of 43S pre-initiation complex (PIC) carrying eIFs 1, 1A, 2, 3, 5 and the first Met-tRNA_i to the cap-binding factor eIF4E and its partners, scaffolding protein eIF4G and helicase protein eIF4A, in the eIF4F complex which is previously bound to the capped 5' end of the mRNA (Fig 1-2). After forming a complex composed of proteins and RNA molecules on the mRNA, also known as 48S PIC, the machinery starts scanning the mRNA to encounter consecutive triplets as they enter the P-site for complementarity to the anticodon of Met-tRNA_i (Fig 1-2). In this complex, eIF2 anchors the Met-tRNA_i by hydrolyzing GTP, and subsequently, alignment of the Met-tRNA_i with the first suitable AUG activates the arrest of scanning and disassociates eIFs. Once eIF2-GDP and other eIFs are released, the large ribosomal subunit (60S) joins the translation machinery to form a fully formed ribosome called 80S initiation complex (Fig 1-2) which is ready to enter elongation step and receive the correct aminoacyl-tRNA into the A (aminoacyl) site to synthesize the peptide bond one at the time which eventually gives rise to a fully synthesized protein (Pestova *et al.*, 2007; Zhao *et al.*, 2017). This process can also be initiated free of 5' cap-structure by direct binding of ribosome to specific region of

mRNA called Internal Ribosome Entry Site (IRES) (López-Lastra, 2005; Johnson *et al.*, 2017).

Figure 1-2: Schematic representation of eukaryotic translation initiation phase underlying the main steps. Translation initiation is the phase at which ribosomal subunits, along with translation initiation factors, interact with an mRNA and upon reaching the first appropriate start codon (AUG), initiate the translation.

1.2.2 Elongation of translation

The elongation step of translation gives rise to formation of a polypeptide chain. Right after formation of 80S ribosomal initiation complex, the ribosome hydrolyzes GTP to move along the mRNA from 5' to the 3' direction utilizing elongation factors (eEFs) to serially incorporate the appropriate aminoacyl-tRNAs in line to bind new amino acids based on the codon assortments on the mRNA to synthesize a new polypeptide chain (Fig 1-3). The aminoacyl-tRNAs carrying the appropriate amino acid, first enters from the Aminoacyl site (A-site) and subsequently is translocated into the P-site, leaving the A-site empty for the next aminoacyl-tRNA to enter (Fig 1-3). This process then shifts the ribosome down the mRNA by three nucleotides, placing a new appropriate mRNA codon into the empty A-site according to the codon on the mRNA (Fig 1-3) (Kapp and Lorsch,

2004; Zhao *et al.*, 2017). Elongation, relative to other translation steps, is more conserved in most eukaryotic organisms. This process requires two main eukaryotic Elongation Factors (eEFs); eEF1A and eEF2. eEF1A interacts with aminoacyl-tRNA to facilitate its binding to the A-site of the ribosome. As soon as a charged tRNA enters the A-site, the large subunit facilitates a peptide bond of the amino acids which also utilizes the peptidyl transferase activity (Fig 1-3). The translocation of aminoacyl-tRNA from the A-site of the ribosome to the P-site is utilized by the activity of eEF2 and at the same time, the A-site of the ribosome is moved to the next codon on the mRNA in a GTP-dependent manner (Fig 1-3). eEF3 is an ATPase, that in yeast, hydrolyses ATP to facilitate the binding of aminoacyl-tRNA-eEF1A-GTP to the A-site and release the deacylated tRNA from the E-site (Fig 1-3) (Sandbaken *et al.*, 1990; Kapp and Lorsch, 2004; Andersen *et al.*, 2006; Burglová *et al.*, 2018). This procedure is repeated until a stop codon is encountered which then activates the termination process.

Figure 1-3: Schematic representation of eukaryotic translation elongation phase underlying the main steps. The 80S ribosome in correlation with elongation factors and aminoacyl-tRNAs move on an mRNA in 5' to 3' direction to convert genetic triplet codes into polypeptide chain.

1.2.3 Termination of translation

Upon reaching one of the three in-frame stop codons (UAA, UAG and UGA) at the A-site of the ribosome, translation machinery terminates the synthesis of the growing polypeptide chain. In eukaryotic cells, two classes of eukaryotic Release Factors (eRFs); eRF1 and eRF3, (Fig 1-4) interact with each other to form a heterodimer that mediates termination. Previous studies demonstrated that eRFs can mimic tRNA's shape and as soon as eRF1 encounters a stop codon on an mRNA, it enters the A-site of the ribosome, and subsequently triggers a biochemical reaction to form an ester bond with the peptidyl-tRNA in the P-site to release the polypeptide chain from the translation machinery (Kissele and Buckingham, 2000; Chavatte *et al.*, 2003). Immediately after this interaction, eRF3 by hydrolyzing GTP, binds to the ribosome, and stimulates the release of eRF1 (Grentzmann *et al.*, 1998; Berk and Zipursky, 2000; Raimondeau *et al.*, 2018). The interaction between the eRFs and translation machinery activates the ribosomal large subunit peptidyl transferase center, which then initiates the hydrolysis of the peptidyl-tRNA bond and therefore detaches the polypeptide chain (Kissele and Buckingham, 2000; Chavatte *et al.*, 2003). In this process, eRF1 and eRF3 play the most crucial role for optimum efficiency of translation termination pathway (Fig 1-4). As soon as the process of protein synthesis has terminated, the complete ribosome is either disassembled and its subunits recycled for use on a different mRNA or it stays attached on the mRNA to form a loop and translate the same mRNA again. Reassembly of the ribosome to translate the same mRNA can aid in more efficient and faster translation (Chavatte *et al.*, 2003; Raimondeau *et al.*, 2018).

Figure 1-4: Schematic representation of eukaryotic translation elongation phase underlying the main steps. Termination step is achieved when a stop codon is detected. Release factors disassemble ribosome to its subunits and the fully synthesized polypeptide chain is released.

1.2.4 Regulation of translation initiation

Irrefutably, regulation of protein synthesis exerts a reflective effect on general gene expression. If identical quantities of two different mRNA transcripts are present in the cell, they may be translated at different levels. The recruitment of the ribosomal subunits to the mRNA and formation of the 80S ribosome play the most crucial role in this phenomenon which opens massive platform for manipulation of gene expression at translational levels. As mentioned above, ribosomes are attached to the mRNA at translation initiation via intensive interactions between several eukaryotic initiation factors (eIFs) (Sonenberg and Hinnebusch, 2009; Hinnebusch *et al.*, 2016). The rate that an mRNA molecule is translated holds a crucial role in majority of essential biological processes such as the response to biological signals, environmental stresses, cell growth and cellular development (Hershey and Merrick, 2000).

Most vertebrate mRNAs are functionally monocistronic and contain a cap-

structure at their 5' ends. The start codon used as the initiation site for protein translation is preceded by a 5' un-translated region (UTR) in which nucleotide structure, composition, and length are thought to direct the efficiency by which translation of a given mRNA is initiated (Hershey and Merrick, 2000; Hinnebusch *et al.*, 2016). In majority of eukaryotic mRNAs, the recruitment of ribosome to the mRNA takes place on the 5' UTR which makes this region along with start codon choice play a critical role in the regulation of translation efficiency and consequently shaping the cellular proteome profile (Hinnebusch *et al.*, 2016). For the most part, the factors that are involved in the recruitment of 43S PIC on the mRNA and formation of 48S PIC constitute the limiting step in regulation of translation initiation. For example, during stress conditions, the phosphorylation of eIF2 plays a crucial role in regulation of translation initiation by converting eIF2-GDP into a competitive inhibitor of the 5-subunit GEF (guanine nucleotide exchange factor), eIF2B, and decreasing ternary complex (TC) assembly (Sonenberg and Hinnebusch, 2009; Hinnebusch *et al.*, 2016). Most eukaryotic mRNAs carry a 5' terminal nuclear modification (7-methylguanosine cap), added to the 5' end of the mRNA, which integrates several important functions and affects RNA splicing, transport, stabilization and translation (Ziehr *et al.*, 2015). During the assembly of the 43S PIC on the mRNA, the cap structure, bound to eIF4F complex via eIF4E, serves as a "molecular tag" that facilitates the landing spot to which the 43S PIC is recruited (Ramanathan *et al.*, 2016).

The interaction between eIF4E and eIF4G is essential for the formation of the eIF4F complex hence subsequently for cap-dependent translation initiation in eukaryotic cells (Jaiswal *et al.*, 2018). Protein synthesis can also be regulated based on the assembly

of the eIF4F complex which is strongly controlled by a large family of translational repressors known as eIF4E-binding proteins (4E-BPs) (Grüner *et al.*, 2018). This kind of regulation/inhibition can be overcome by viruses of the family *Picornaviridae* (e.g. *poliovirus*, *coxsackievirus*, human *rhinoviruses* etc.), containing a single stranded RNA genome which is transcribed to a single viral ORF. Successful viral replication is majorly dependent on efficient translation of the RNA genome into the polyprotein products. In order to deliver maximum translational efficiency, most picornaviruses inhibit the host translation machinery and at the same time allowing viral RNA to be actively translated by the full potential of the host translation machinery. Majority of *picornaviruses* can accomplish this achievement by cleaving the two isoforms of the cellular eIF4G, eIF4GI and eIF4GII into two different domains hence effectively non-functional (Foeger *et al.*, 2003, Aumayr *et al.*, 2017). As a consequence of incomplete form of eIF4G, eIF4F complex loses its ability to bind to eIF3 (the protein that is linked to the 40S ribosomal subunit). This in return causes the host cells to lose their ability to join the 43S PIC to the capped mRNA whereas the translation of viral RNA remains active as a result of IRES-mediated translation (Martinez-Salas and Ryan, 2010; Aumayr *et al.*, 2017).

In contrast to canonical cap-dependent translation, IRES-mediated translation occurs independently of a cap structure (Leppek *et al.*, 2018). This alternative pathway can be used preferentially when one or more specific initiation factors are lacking. In eukaryotic cells, this pathway is also often used to initiate the translation of specific mRNAs when general translation initiation is compromised (Faye and Holcik, 2014). Therefore, it is thought that this process plays an important role for regulation of gene expression in a number of stress conditions such as pathogen infection (Mohr &

Sonenberg, 2012), hypoxia (Young *et al.*, 2008) and heavy metal conditions (Rai *et al.*, 2003; Moteshareie *et al.*, 2018).

1.2.5 Internal Ribosome Entry Site (IRES) and its activation

For decades the cap-dependent method of translation initiation was considered the only route by which translation of eukaryotic mRNAs could be initiated. However, in recent decades certain unique mRNAs, predominantly in viruses, have shown to be able to bypass the canonical initiation scanning mechanism and a subset of eIFs by using internal structures formed at their mRNA levels to recruit the 40S ribosomal subunit directly to the mRNA (Sonenberg and Hinnebusch, 2009). Translation of RNAs that form IRES structures, can be initiated without the need for cap-binding proteins by direct recruitment of ribosome to a specific region of an RNA usually in 5' UTR, known as IRES. This is a cap-independent process which mediates 5'-end-independent ribosomal recruitment to internal locations on the mRNA (López-Lastra, 2005). Such internal sequences were first discovered in 1988, in *picornaviruses*, such as RNAs of *poliovirus* and *encephalomyocarditis* virus (Pelletier and Sonenberg, 1988). These viruses utilize IRES structures on their RNA to efficiently hijack the host translation machinery for maximum expression and replication of their genetic material. This also aids viruses to overcome the inhibition of cap-dependent translation initiation during viral infection (Pelletier and Sonenberg, 1988). These viruses encode for proteases that cleave both isoforms of eIF4G. Separating eIF4G into N-terminal eIF4G/eIF4E/PABP and C-terminal eIF4G/eIF4A/eIF3 complexes leads to uncoupling the cap-recognition from ribosome-binding and helicase functions of eIF4F (Gradi *et al.*, 1998). In addition, it is thought that sequestration of eIF4E by eIF4-BPs is increased as a result of virus infection (Gingras *et*

al., 1996). Both mechanisms strongly reduce the activity of eIF4F in cap-mediated initiation in host mRNAs during viral infections (Kieft, 2008; Johnson *et al.*, 2017; Lepppek *et al.*, 2018). Soon after the discovery of IRES-mediated translation of *poliovirus* RNAs, the first IRES element in eukaryotic cellular mRNA was discovered in *poliovirus*-infected cells at the time when the major canonical translation of host cell mRNAs were inhibited (Sarnow, 1989). In more recent studies, additional IRES-containing cellular mRNAs have been identified in *poliovirus*-infected cells (Johannes *et al.*, 1999) and current studies suggest that approximately, 10% of the eukaryotic mRNAs are translated independent of cap structure or full formation of eIF4F (Stoneley and Willis, 2004).

Based on their requirements for non-canonical translational factors and their interactions with eIFs, and 40S ribosomal subunit, structurally-related viral IRESs are generally divided into four different types (Jackson *et al.*, 2010; Johnson *et al.*, 2017). Initiation of translation on type 1 and type 2 IRESs involves their specific binding to the p50 domain of eIF4G, which is enhanced by eIF4A. The eIF4G–eIF4A complex recruits 43S complex without the involvement of eIF4E (Fig 1-5) (Jackson *et al.*, 2010; Johnson *et al.*, 2017). Type 3 IRESs interact with the eIF3 and 40S subunit components of 43S complexes and directly attach 43S complexes to the initiation codon independent of eIF4F, eIF4B, eIF1 and eIF1A (Fig 1-5) (Jackson *et al.*, 2010; Johnson *et al.*, 2017). Type 4 IRESs directly recruit 40S subunits; they initiate translation without eIFs or Met-tRNA_i (Fig 1-5). Hence, this type of IRES-mediated translation initiation might be resistant to cellular regulatory mechanisms, such as eIF2 phosphorylation (type 4 viral IRES) and/or eIF4E sequestration (all four types of viral IRESs) (Fig 1-5) (Johnson *et al.*, 2017). It is noteworthy that a recent study has discovered the first case of a cellular IRES component

that is capable of direct recruitment of 40S ribosomal subunit without the need for any of the canonical eIFs. The IRES element of c-Src kinase mRNA is similar to hepatitis C virus-like IRESs based on the requirements for initiating translation (Allam and Ali, 2010). In addition to multiple canonical eIFs, majority of IRESs also require extra factors called IRES trans-acting factors (ITAFs) which are generally RNA-binding proteins that are thought to stabilize the optimal three-dimensional IRES conformation as PTB and ITAF45 are shown in type 2 IRES (Fig 1-5) (Johnson *et al.*, 2017). Several IRESs (e.g. eIF3d, *APAF1*, HSP70 etc.,) also require specific short regulatory RNA structures such as circular RNAs (circRNAs) for proper initiation of IRES-dependent translation (Wolfe *et al.*, 2014; Iwasaki *et al.*, 2016; Leppek *et al.*, 2018).

Figure 1-5: Schematic representation of four different types of viral IRES structures. Viruses that contain IRES structures at their RNA levels, can generally form four distinguishable internal structures based on their requirements for the initiation and IRES factors.

Unlike viral IRESs that have been classified to four types, eukaryotic IRES elements are so diverse that makes them very difficult to cluster into different groups (Komar, and Hatzoglou, 2011). Each cellular IRES holds a unique structure and requirements for specific ITAFs. However, same as viral IRESs, eukaryotic IRES elements also interact with general components of the translational machinery such as canonical initiation factors and 40S ribosomal subunit in addition to certain specific and non-specific ITAFs. These interactions are thought to be most likely responsible for proper positioning of the ribosomal P-site on the initiation codon (Yamamoto *et al.*, 2017). Several cellular IRESs may utilize the interaction between the ITAFs and components of the translational machinery to modulate a landing ground in the vicinity of initiation codon for 40S ribosomal subunit, using a mechanism called “land and scan mechanism” (e.g. picornavirus) (Belsham, 2000; Johnson *et al.*, 2017). Other cellular IRESs, may modulate ITAFs for a Shine-Dalgarno-like interaction between the 18S ribosomal RNA (rRNA) and the IRES structure on the mRNA (Meng *et al.*, 2010). For example, a short IRES consist of nine nucleotide (nt) of the human homeodomain *GTX* mRNA (Chappell *et al.*, 2004) and a 90 nt IRES of the human proto-oncogene *IGF1R* mRNA operate on a Shine-Dalgarno-like mechanism (Meng *et al.*, 2010). Previous studies suggest that a significant proportion of cellular translation initiation is driven from IRES-mediated initiation (Johannes *et al.*, 1999; Spriggs *et al.*, 2005; Stoneley and Willis, 2004). Identification and investigation of the factors that mediate these interactions and the mechanisms that govern the recruitment of the translational machinery to IRES region of an mRNA have been the main goal of many studies in the last couple of decades (Chappell *et al.*, 2004; Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018). Previous studies

suggest that approximately translation of 10–15% of cellular mRNAs are initiated free of 5' cap, from which 3-4% have been verified to contain IRES element (Spriggs *et al.*, 2008). Polyribosome analysis suggest that 3–5% of cellular mRNAs are translated very efficiently under stress conditions via IRES-mediated translation or similar ways (Spriggs *et al.*, 2008; Schrufer *et al.*, 2010). Studies suggest that majority of cellular mRNAs that contain IRES elements encode for transcription factors, growth factors and transporters however after over two decades, few examples of ITAFs have been well characterized (Lacerda *et al.*, 2017).

As mentioned before, regulation or in some cases inhibition of translation most likely takes place at the highly controlled initiation step of translation (Leppek *et al.*, 2018). Under stress conditions, initiation step is generally regulated through cleavage of the cap-binding protein eIF4E or the scaffolding protein eIF4G (Komar and Hatzoglou, 2011). Stress conditions can also influence the delivery mechanism of Met-tRNA_i. These conditions mainly cause phosphorylation of the eIF2 alpha subunit (eIF2 α) (e.g., viral infection, nutrient limitation, ER stress, etc.,) (Wek *et al.*, 2006). Since under these stress conditions, certain mRNAs can undergo IRES-mediated translation, they must resist any regulation of eIF4E and eIF4G (Prévôt *et al.*, 2003). In addition to these two important initiation factors, several cellular IRES elements also show multiple rate of resistance to inhibition of protein synthesis caused by phosphorylation of eIF2 α (e.g. s-Src, cat-1, N-myc, etc.,) (Komar and Hatzoglou, 2005; Tinton *et al.*, 2009). Previous studies suggest that the reduction of translation caused by phosphorylation of eIF2 α and eIF2A can significantly influence cellular IRES-mediated translation (Reineke *et al.*, 2011; Myasnikov *et al.*, 2009).

More than two decades of investigation into cellular and viral IRES-mediated translation, strongly suggest that under physiological, pathophysiological and stress conditions that can decrease or inhibit cap-dependent initiation such as endoplasmic reticulum (ER) stress, hypoxia, nutrient limitation, mitosis and cell differentiation, IRES-mediated translation takes over and mRNAs containing IRES become more competitive for the available pool of translational components and ITAFs (Komar and Hatzoglou, 2005; Lewis and Holcik, 2008; Lacerda *et al.*, 2017). It is striking that many of the cellular mRNAs that contain IRES elements encode proteins that are involved in protection of cells from stress or, alternatively, induction of programmed cell death (apoptosis) (Mokrejš *et al.*, 2009; Samanfar *et al.*, 2017). In chapter four of this study, we identified IRES-containing mRNAs that function as ITAFs and may play essential roles for proper activation of specific IRES-mediated mRNAs. This strongly suggests that cellular IRES-mediated translation plays a key role in the fate of a cell and cellular decision making under a variety of stress conditions (e.g. Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018). In these stress conditions that inhibit cap-dependent translation, different cellular IRESs may impose various responses to these conditions by regulating the expression of cellular ITAFs. They are responsible for sensing changes in cellular metabolism and modulate their expression accordingly which influence IRES activity (Komar and Hatzoglou, 2005; Spriggs *et al.*, 2005; Lewis and Holcik, 2008). Cellular ITAFs can be independent factors and play no role in canonical translation or they can perform multifunction and be involve in various processes (Komar and Hatzoglou, 2011; Yamamoto *et al.*, 2017). Interestingly, majority of cellular ITAFs belong to the group of heterogeneous nuclear ribonucleoproteins (HnRNP A1, C1/C2, I, E1/E2, K and L) which

are suggested to move between the cytoplasm and the nucleus (Spriggs *et al.*, 2005; Lewis and Holcik, 2008; Komar and Hatzoglou, 2011). Cellular ITAFs are also known to participate in multiple cellular processes such as RNA export and/or splicing (Komar and Hatzoglou, 2011; Lacerda *et al.*, 2017). Nevertheless, most importantly ITAFs are commonly known to regulate the affinity of interactions between IRESs and components of the translation machinery (40S ribosomal subunit and canonical eIFs). Despite the extensive efforts over the past couple of decades, the precise mechanism(s), governing ITAF functions is not well-understood. However, by far three hypotheses exist; 1) ITAFs are able to modify IRES structures to facilitate binding with different levels of affinity for components of the translation machinery, 2) they can form bridges between the ribosome and the mRNA strand which is in addition to the general bridges formed by canonical initiation factors and 3) they can be replaced by canonical factors in forming bridges between the ribosome and the mRNA (Komar and Hatzoglou, 2011; ; Yamamoto *et al.*, 2017).

The mechanisms of action in viral IRESs is better understood than cellular IRESs, the cellular IRESs are largely unknown hence they need more thorough investigations (Komar and Hatzoglou, 2011). It is previously suggested that the activity of cellular IRES elements might be based on short structural motifs on the IRES region of the mRNA (Mitchell *et al.*, 2005; Johnson *et al.*, 2017) with the assistance of eIFs or RBPs, serving as ITAFs (Komar and Hatzoglou, 2011; Faye and Holcik, 2014). Based on previous studies, ITAFs showed to be essential for proper initiation of IRES-mediated translation. Studies suggest that several ITAFs may be common between viral and cellular IRESs however only a few ITAFs are well characterized (Lewis and Holcik, 2008; Faye and

Holcik, 2014; Yamamoto *et al.*, 2017). The interactions between IRES element and ITAFs may either stabilize an unstable IRES structure or they can give rise to a conformational change to the IRES regions that facilitate the recruitment and correct positioning of the translation machinery (Johnson *et al.*, 2017). It is suggested that structural modifications in RNA motifs of many cellular IRESs can activate them followed by cellular conditions that can trigger such changes in which case RBPs most likely act as RNA chaperones. For example, such conformational changes and process can be traced in cells with viral infection or apoptosis, the proto-oncogene *MYC*/IRES is activated by genotoxic stress caused by such conditions (Cobbold *et al.*, 2010). Other examples include BCL-2 associated athanogene 1 (*BAG1*) (Pickering *et al.*, 2003; Pickering *et al.*, 2004) and apoptotic peptidase activating factor 1 (*APAF1*) mRNAs (Mitchell *et al.*, 2003).

Extensively, cellular IRESs (~100–200 nt) are known to appear shorter in length compare to viral IRES elements (~300–600 nt) (Komar and Hatzoglou, 2005). They are less stable (low G and C content) and more diverse in their secondary structures (Komar and Hatzoglou, 2005; 2011). In contrast to viral IRES elements, there has not been identified any known eukaryotic IRES element to possess common structural motifs (Baird *et al.*, 2007). Previously, cellular IRES elements were identified based upon the use of a bi-cistronic mRNA which was lacking enough sensitivity (Pelletier and Sonenberg, 1988). However, in recent years, based on the bi-cistronic vectors, expressing the firefly, β -galactosidase and renella luciferase enzymes, the sensitivity for expression of both cistrons has been significantly increased which aided in the detection of very low levels of protein synthesis under mimicked cap-independent conditions. Due to the high

sensitivity of these assays, significant (several-fold) increase in the expression of the second cistron could be observed which may generally be due to the lack of true IRES (Anthony and Merrick, 1991). Identification of cellular IRES elements also holds several technical difficulties including alternative splicing and/or cryptic promoters in the IRES sequence which results in a monocistronic mRNA for the second cistron, in which case the small fold increase usually makes it difficult to truly claim a genuine IRES element (Kozak, 2001; Merrick, 2010; Komar et al., 2012; Yamamoto et al., 2018). Molecular biologists, in the past couple of decades have been actively trying to develop a variety of alternative methods and assays to address whether expression from the second cistron is truly an IRES-dependent synthesis. Practically, most scientists in this field, used at least two methods as proof of the existence of an IRES element.

In the recent years, with the development of new concepts in protein synthesis, including cap-assisted internal initiation and 5'-end and scanning-dependent but cap-independent initiation mechanisms, the controversies in the identification of true IRES has become even more complicated (Miller et al., 2007; Andreev et al., 2009; Shatsky et al., 2010). Based on the fact that all cellular mRNAs are expected to forgo through the process of capping, the main concerns remain whether the cap-structure may indeed assist internal initiation and/or whether IRES-containing mRNAs may be translated via both mechanisms. Certainly, concept of IRES relies on the utilization of cap-independent initiation of translation. So far, no evidence, supporting any increase in the addition of a cap structure under translational control of cellular IRES elements. A process similar to what currently is referred to as “shunting” (Yueh and Schneider, 2000) is also proposed for some cellular IRESs where it is suggested that an mRNA may be recruited to the 40S

subunit and then bypasses extensive secondary structures and bind directly at the IRES-AUG border.

Presently, the process by which and reasons for why a certain sequence or structure may act as a cellular IRES. It is suggested that the folded/ structured RNAs adopt RNA protein configurations similar to those of the tRNAs and thus may pose some affinity for the internal ribosomal sites (A, P or E site) as previously observed for several viral IRESs (Komar et al., 2012). It is alternatively suggested, that the IRES element may facilitate the binding with high affinity to eIF4F which in return mimics cap structure as seen in poliovirus or EMCV RNA (Fig 1-5). Another logical proposal suggests the existence of proteins that can specifically bind to the IRES elements that can form an mRNP with the ability to mimic one of the normal protein/RNA complexes that has high affinity for the ribosome (Komar et al., 2012). Evidently, the lack of more definitive data opens more room for much speculation (Jackson, 2013). More data and new ways of looking at the cellular IRESs and the mechanisms that governs this process is clearly lacking. Probably looking to the factors that can influence this process may be a good approach. Researchers have so far demonstrated the reconstitution of the translation initiation complexes of viral IRESs, using purified cellular components. However, such verification has never been reported for any of the cellular IRESs so far (Kolupaeva et al., 2003; Lomakin et al., 2000; Pestova et al., 1996, 1998) which fuels the skepticisms on the authenticities of many cellular IRESs (Kozak, 2005; Shatsky et al., 2010). This could be due to the lack of our understanding of many essential (yet undiscovered) ITAFs that are yet to achieve successful reconstitution.

After more than two decades of investigating and studying viral and cellular IRESs, the mechanism of viral IRESs becoming better understood however contrary to viral IRESs (Hellen, 2009; Balvay, *et al.*, 2009; Filbin and Kieft, 2009) very little is currently known about the mechanisms that govern the function of cellular IRESs. To address this lack of knowledge we used baker's yeast *S. cerevisiae* as a model organism to investigate cellular IRESs. Several yeast mRNAs also possess IRES elements (e.g. *YAP1*, *URE2*, *HSP82*, *FLO8*, *GPRI* etc.), which makes it an ideal organism to investigate cellular IRESs. In this project, we investigated multiple genes that are previously suspected to form IRES on their mRNA molecule. Previous studies also showed the ability of yeast to serve as an ideal model organism to study cellular IRESs (Shaikho *et al.*, 2016; Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018). Using baker's yeast, Samanfar *et al.*, in 2017 identified two genes; Duplication Of Multilocus 34 (DOM34) and Ribosomal Protein of the Large subunit 36A (RPL36A) that potentially serve as ITAFs for *HSP82*-IRES during apoptosis conditions such as heat shock or exposure to acidic acid. The main focus of this project is based on yeast Ureidosuccinate Transport 2 (*URE2*) gene which possesses an IRES element on its mRNA (Komar *et al.*, 2003). Ure2p is homologous to mammalian Glutathione S-transferases protein and plays an essential role in stress response to toxicity of heavy metals and metalloids.

1.2.6 Ureidosuccinate Transport 2 (*URE2*) contains an IRES element

URE2 mRNA possesses an IRES structure allowing for cap- and eIF4E-independent synthesis of its protein (Fig 1-6). The cap-dependent translation of *URE2* gene is 94 amino acids larger than cap-independent translated form that possesses prionogenic properties and is often referred to as the Ure3p form. The cap-independent

translated form of *URE2* mRNA is N-terminally truncated and starts from amino acid 94 to 354. The truncated form of Ure2p lacks the prion-forming domain that affects Ure3p prion propagation in *S. cerevisiae* cells. The activity of the *URE2*-IRES is increased when eIF4E and eIF2A activities are down-regulated (Reineke *et al.*, 2011). The Ure2p in *S. cerevisiae* has been studied in three different aspects: as the protease-resistant prion-like altered form of its protein (Ure3) (Wickner *et al.*, 2000); as a negative regulator of GATA-dependent transcription (Cooper, 2002); and as a glutathione S-transferase (GST) protein (Choi *et al.*, 1998; Bousset *et al.*, 2001). In *S. cerevisiae* the Ure2p has glutathione peroxidase activity and is homologous to mammalian glutathione S-transferases. The resistance of *S. cerevisiae* to a wide range of heavy ions is carried out by glutathione S-transferase product. Gene deletion of *URE2* exhibits hyper-sensitivity to various metals and metalloids including cadmium and arsenic (Todorova *et al.*, 2010; Moteshareie *et al.*, 2018).

Figure 1-6: Internal structure of *URE2* mRNA. *URE2*-IRES is approximately 100 nt and forms 4 hairpin loop structures on its mRNA. *URE2* can be translated free of the cap-binding factor (eIF4E) and it is resistant to cleavage of eIF4G.

1.2.7 Why study cellular IRESs

IRESs can be adopted as translational enhancers that naturally occur in a series of cellular and viral mRNAs, facilitating internal initiation of translation when designed between the genes of interest enabling the design of multicistronic expression cassettes. These cassettes resemble bacterial operons and are able to mediate translation of multiple genes synthesized by the same mRNA (Renaud-Gabardos *et al.*, 2015). Due to the strong efficiency of picornavirus IRES, it is used for the development of most IRES-based vectors, compared to cellular IRESs. It is suggested that the low efficiency of cellular IRESs is because of the cell and tissue specificity of the cellular IRESs (Créancier *et al.*, 2000). By using the tissue-specific cellular IRESs, transgenes expression can be controlled. This feature can also be coupled with transcriptional enhancers upstream of promoters to enhance translation to create vectors with increased safety (Renaud-Gabardos *et al.*, 2015).

In addition to biotechnology applications of IRESs, it is imperative to study cellular IRESs since many diseases and environmental stressors are functionally linked to IRES activities. As explained before, under stress conditions, canonical cap-dependent form of translation initiation maybe inhibited where IRES-dependent translation is not affected (Johnson *et al.*, 2017). Vital cellular mRNAs, responsible for proteins that are involved in the stress response, especially apoptosis and hypoxia, naturally contain IRES elements to overcome such stress conditions. For example, two yeast mRNAs that encode for two heat shock proteins; *HSP82* and *HSC82* are involved in apoptosis (Silva *et al.*, 2013). Both genes form IRES structures on their mRNAs (Silva *et al.*, 2013; Samanfar *et al.*, 2017). Another example is the hypoxia-induced factor 1 α (*HIF1 α*) that is expressed in

response to hypoxia which is responsible to induce transcription of a gene family containing a hypoxia responsive element (HRE) based on their promoters. The mRNA of *HIF1 α* also contains an IRES element, allowing its expression under oxidative stresses where global cap-dependant translation is compromised (Lang *et al.*, 2002; Braunstein *et al.*, 2007). IRES-mediated translation of *HIF1 α* is under the control of a general ITAF with multifunction called the pyrimidine tract binding protein. This ITAF is well-known for its involvement in multiple IRESs (Schepens *et al.*, 2005; Romanelli *et al.*, 2013).

In the last decade IRESs have also been utilized in certain biomedical applications. For example, a therapeutic application of IRESs is proposed by Qy Liu in 2015 to treat Alzheimer's disease (AD). It was previously shown that β -amyloid ($A\beta$) plaques and tau tangles aid in the biosynthesis of AD. It was also suggested that amyloid precursor protein (APP) is required for the biosynthesis of $A\beta$ to activate the process of AD (Smith and Perry, 1998). The over accumulation of $A\beta$ under certain pathological conditions can disturb the normal function of *APP*. Therefore, it can be hypothesized that reduction in the expression of APP during the pathophysiological stress conditions, perhaps could treat AD. The *APP* mRNA is produced through both canonical cap-dependent and IRES-mediated translation hence it was speculated that the level of *APP* in the cells may not be decreased mainly due to self-protection, likely because *APP* can be translated through IRES-mediated translation (Beaudoin *et al.*, 2008). This leads to a conclusion that the blockage of IRES-mediated translation of *APP* might be a new high-level specificity and inoffensive treatment for AD. It is noteworthy that knowing the specific mechanisms and ITAFs that govern IRES-mediated translation of *APP* is significantly important in this case.

Gene therapy as a novel therapeutic target has received a considerable attention over the last few decades, however being in its infancy, it poses a lot of technical challenges before it can be a practical approach to treating diseases. For example, better ways to deliver genes and target them to particular cells must be found. We must also ensure that new genes are precisely controlled by the specific tissues in the body. Combining the specific featural advantages that utilizing IRESs propose with the known knowledge of gene therapy, we can overcome some of the proposed challenges. Several studies in the past decade have validated this concept by using a mixture of two vectors to transfer two different genes simultaneously. Several animal models have already been tested, using different combinations of angiogenic or anti-angiogenic factors, which is particularly demonstrated in the field of cardiovascular diseases and cancer, with promising therapeutic benefits (Chae *et al.*, 2000; Scappaticci *et al.*, 2001; Zhang *et al.*, 2007; Kupatt *et al.*, 2010). For example, using IRES-mediated translation, the combination of vascular endothelial growth factor A (*VEGFA*) and platelet-derived growth factor subunit B (*PDGFB*) utilized for therapeutic purposes and successfully induced angiogenesis in both ischemic heart and leg (Abmayr *et al.*, 2005).

1.3 Systems biology

In the past few decades, we have witnessed a significant acceleration in our understanding of biological systems at the molecular level. Improvements in molecular genetics methodologies have allowed us to sequence the entire human genome in addition to a large number of other species. With the aid of computational tools, we are now one step closer to complete the entire map of life. The more we understand about intracellular regulatory networks, we face new challenges of integrating all available molecular data to

quantitatively comprehend the behavior of a cell or organism at the biological systems level (Palsson and Palsson, 2015). Systems biology offers a new platform for the research effort that delivers the scientific basis for successful experiments in synthetic biology. This relatively new born and exciting field of biology is based on the comprehensive investigation of the molecular cross-communications with other cellular processes in living organisms, both synthetic and natural (Breitling, 2010; Palsson, 2015). Systems biology also tries to contribute to the documentation of simplifying overall principles and designs that are recurring features in living and engineered organisms, and the integration of the known biological knowledge in simplified complex models of the regulatory networks that characterize dynamics of living systems (Breitling, 2010).

Over the past few decades, scientists in systems biology have been trying to sort out the puzzle of life by investigating the genetic information and their systematic functions using different model organisms that are well adopted to laboratory conditions including *Drosophila melanogaster*, *Caenorhabditis elegans*, *S. cerevisiae*, *E. coli* and several other organisms (Kholodenko *et al.*, 2015). Function of individual genes and their relationship with other cellular components in living organisms have been the subject of vigorous studies since the discovery of DNA by Watson and Crick in 1953. In the beginning of this journey, scientists used classical genetics to investigate one gene or a gene family at a time which combines isolated results to better understand the dynamics of a cell (Palsson and Palsson, 2015).

Although these methods are very resourceful, they do not impose the possibility to accurately model the anatomy of a living organism or a cell as a whole system. This is

due to the lack of comprehensive coverage for global communications between components of a cell and interacting pathways. In addition, these methods are labor and resource intensive and, in most cases, do not accurately represent the biology of a cell or an organism as a whole system. These limitations signify the absence of genome-scale investigations and high-throughput experimental tools to precisely assemble models of biological systems at molecular levels (Kholodenko *et al.*, 2015) to characterize the dynamics that govern an individual cell or living organisms as a system at the molecular levels.

Over the last two decades, a growing number of high-throughput techniques have been developed to provide systematic approaches for global cell measurements in the field of genomics and proteomics (Rigaut *et al.*, 1999; Tong *et al.*, 2001; Parson *et al.*, 2004; Hillenmeyer *et al.*, 2008; Palsson and Palsson, 2015). A significant increase in the availability of quantitative modeling of biochemical reaction networks of various model and non-model organisms have also significantly impacted the field of molecular biology and genetics (Palsson and Palsson, 2015). During this time, a wide variety of high-throughput approaches have been improved and developed to investigate the function of genes and proteins and their cross-communications with other pathways in a systematic manner at transcriptome, proteome and metabolome levels (Oliver *et al.*, 2002; Samanfar *et al.*, 2014; Babu *et al.*, 2018). Considering the amount of data produced by these large-scale approaches, necessity of computational power is unavoidable and must be paired with molecular biology. This is where systems biology with a goal to understanding living organisms at the systems level can be applied. Systems biology combines quantitative systematic data on individual components of a cell including interacting

partners in order to understand the resulted emergent behaviors. In addition, quantitative high-throughput methods provide more precise systematic predictions that will offer novel bioengineering approaches for biotargets and drug discovery (e.g. Kholodenko *et al.*, 2015, Pitre *et al.*, 2006; Schoenrock *et al.*, 2017; Kazmirchuk *et al.*, 2017). The capability of understanding and measuring genome-wide, transcriptome-wide or proteome-wide systems is perhaps the single utmost force giving rise to systems biology. Systems biology not only provides large-scale quantifications, but also is the driving force behind the hypothetical philosophy for experimental analysis and design (Kholodenko, *et al.*, 2015). Conclusively, systems biology in addition to large-scale studies that are focused on discoveries, provides a framework for using the experimental data combined with computational force to perform predictive approaches.

One of the most common model organisms currently utilized for large-scale investigation at systems level of eukaryotic cells is the budding yeast, *S. cerevisiae*. This laboratory adopted fungal organism has been vigorously studied and subjected to pioneering genome-wide (e.g. Goffeau *et al.*, 1996), transcriptome-wide (e.g. Ghaemmaghami *et al.*, 2003), and proteome-wide (e.g. Krogan *et al.*, 2006; Samanfar *et al.*, 2013; 2014) experiments. In addition, large-scale experiments in yeast provided a platform for prediction of protein interatomic networks and engineering of novel proteins in other organisms (Pitre *et al.*, 2006; Schoenrock *et al.*, 2015; Schoenrock *et al.*, 2017; Burnside *et al.*, 2018). The integration of data obtained from these approaches can eventually give a framework for modeling biological systems which may explain functional relationships between genes and other cellular communications. For the most part, systems molecular biology is performed by an interdisciplinary team of molecular,

chemical and computational biologists (e.g. Pitre *et al.*, 2006; Schoenrock *et al.*, 2014; Kazmirchuk *et al.*, 2017; Burnside *et al.*, 2018). Several computational investigations in systems biology have been aiming to predict interaction networks within a system; others focus on integrating the available data obtained from different high-throughput techniques to computationally predict, analyze and simulate cellular responses (Jessulat *et al.*, 2011). As mentioned above, in classical molecular biology, functional genomics is defined on basis of individual genes however in the context of systems molecular biology, modern functional genomics describes function of a gene as the interactions of genes and proteins by making use of genome-wide approaches (Palsson and Palsson, 2015).

1.3.1 Functional genomics

The term functional genomics emerged in the late 90's and is given to a field of molecular biology that describes investigations that focus on studying genes and their functions in a systematic manner (Hieter and Boguski, 1997). This field of study attempts to utilize the data produced by genomic projects to describe gene and protein functions. Nowadays, it also covers comprehensive genome wide approaches and analytical tools to identify and characterize novel gene functions and to study gene expression and regulations at molecular levels (Parsons *et al.*, 2006). *S. cerevisiae* is the first eukaryotic model organism whose genome was sequenced, and it was used to pioneer large scale genetic analysis to better understand the biology of a cell (Suter *et al.*, 2006). Genome wide investigations such as transcriptional array analysis (Deris *et al.*, 1997), mass spectrometry analysis for protein-protein interaction (Ho *et al.*, 2002), protein array (Zhu *et al.*, 2001), genetic interaction screens (Tong *et al.*, 2001; 2007) and chemical genetic analysis (Alamgir *et al.*, 2008) are first validated in yeast using well controlled and

reproducible conditions. The wealth of information gathered for more than a decade from detailed analysis of yeast genome combined with the availability of various high-throughput techniques such as gene deletion array (Winzeler *et al.*, 1999) and yeast over expressed array (Sopko *et al.*, 2006) are rapidly increasing the ability of functional analysis tools to systematically investigate the biology of a cell using this organism (Samanfar *et al.*, 2014; Babu *et al.*, 2018).

For example, in 2014, Samanfar *et al.*, utilized yeast non-essential deletion library to investigate genes that affect stop codon bypass. They systematically transferred three shuttle plasmids, pUKC817, pUKC818 and pUKC819, containing three different premature stop codons (UAA, UGA and UAG, respectively) within the LacZ expression cassette, into the yeast non-essential gene deletion array. Transformed array of new strains were subjected to a large-scale β -galactosidase lift assay experiment to analyze the level of β -galactosidase protein produced for each gene deletion strain, where they identified 84 potential gene candidates that may affect stop codon bypass (Samanfar *et al.*, 2014).

Chemical genomics is another approach that in recent years has received a considerable attention to itself because of its ability to investigate functional genomics. Also known as chemogenomics is a new study in the field of molecular biology combined with biochemistry which defines the development of target-specific compounds and the practice of such ligands to globally investigate gene and protein functions. The field of chemical genomics is predicted to play a critical role in the development of novel noninvasive drug discovery and biological research. For example, using yeast deletion set

array, Márquez *et al.*, in 2018 assessed the effects of two commonly used engineered nanoparticles, silver nanoparticles (AgNPs) and zinc oxide nanoparticles (ZnONPs) on yeast cells. A library of approximately 4600 non-essential yeast, *S. cerevisiae*, mutant strains was utilized to infer the genes, whose absence makes *S. cerevisiae* more sensitive to the cytotoxic effects of both compounds (Márquez *et al.*, 2018).

1.4 Aims and Objectives

A range of human diseases such as hyperferritinaemia, hereditary thrombocythaemia and many cancerous conditions in general are caused by alterations in various steps of protein biosynthesis. Over the past few decades, due to its imperative status, the process of translation has been intensively studied. Despite our growing knowledge about this critical process, several genome-wide studies have proposed the existence of other uncharacterized factors that can influence the process of translation (e.g. Krogan *et al.*, 2004; Alamgir *et al.*, 2010; Samanfar *et al.*, 2014; Moteshareie *et al.*, 2018). The precise details of molecular interactions that govern and influence the efficiency and different steps of translation and their cross-communications with other cellular processes have been long baffled scientists and the list of potential players that regulate the process of translation under normal or stress conditions yet continues to grow every year (e.g. Krogan *et al.*, 2004; Holcik and Sonenberg, 2005; Alamgir *et al.*, 2010; Samanfar *et al.*, 2014; Shaikho *et al.*, 2016; Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018).

In eukaryotic cells, translation of cellular mRNAs through cap-independent initiation has received considerable attention during the past two decades (e.g. Créancier

et al., 2000; Pickering *et al.*, 2003; Spriggs *et al.*, 2005; Kieft, 2008; Meng, 2010; Liu, 2015; Yamamoto *et al.*, 2017; Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018). This is partially due to growing evidence that links this process to many important pathological and physiological conditions such as viral infections, apoptosis, hypoxia and various stress conditions including stress caused by exposure to toxic metals (Komar *et al.*, 2011). In addition, IRESs are shown to be an excellent tool for gene therapy and they can also be adopted as translational enhancers to design multicistronic expression cassettes (Kupatt *et al.*, 2010; Renaud-Gabardos *et al.*, 2015). However, there remains a lack of comprehensive understanding of the process of IRES-mediated translation and the list of genes that are involved in this process is rapidly growing. This suggests that there are other uncharacterized genes that can affect the process of IRES-mediated translation. The main goal of this project is to identify and investigate these uncharacterized factors.

Heavy metals and metalloids are toxic elements that impose threat to our health. Major chronic diseases in humans such as renal and cardiovascular diseases, and neurological decline, are also strongly associated with heavy metals and metalloid toxicity (Alwan, 2011). The toxic conditions that heavy metals and metalloids enforce are the main cause of different stresses such as ER stress, hypoxia or apoptosis. Under these stress conditions, cap-dependent translation initiation is suppressed or inhibited where IRES-dependent translation does not seem to be affected (Thakor and Holcik, 2011; Johnson *et al.*, 2017). In yeast, vital cellular mRNAs, responsible for proteins that are essential under toxic metal conditions, appear to contain IRES elements to overcome such stress conditions and inhibition of canonical cap-dependent translation (e.g. *URE2*, *HSP82*, *HSC82*, *FLO8*, *YAP1* etc.). Understanding the genetic factors that influence IRES

mediated translation under stress conditions is an important area of research (e.g. Komar and Hatzoglou, 2005; Spriggs *et al.*, 2005; Lewis and Holcik, 2008; Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018).

Yeast *URE2* is shown to have a glutathione peroxidase activity and can function as a detoxification gene under toxic conditions of heavy metals and metalloids. This gene is also under the translational control of an IRES element (Todorova *et al.*, 2010). Consequently, we hypothesize that there may exist a number of unidentified genetic factors that specifically and/or generally influence the process of IRES-mediated translation of *URE2* in yeast.

Our aim in the current research has been to identify and study novel gene functions that can affect IRES-mediated translation by screening a mutant array of yeast non-essential gene(s). Analyzing the activity of these genes can increase our understanding of the process of translation and possibly its communication with other cellular processes within a cell. In parallel, we utilized new approaches in yeast chemical genomics to analyze the cellular pathways that is specifically influenced by Mn.

Our specific objectives have been as follows:

1. Investigation into the role of four genes; *ITT1*, *RPS1A*, *UGA4* and *YBR062C* in IRES-mediate translation of yeast *URE2*, a key gene involved in metal detoxification in yeast.

2. Screening of the yeast gene deletion library to identify novel genes that specifically or generally affect the process of IRES-mediated translation, using eight

different yeast IRES elements belonging to *HSP82*, *HSC82*, *URE2*, *FLO8*, *GPR1*, *YAP1*, *NCE102* and *HAP4*.

3. Yeast genome-wide investigation to identify novel genes and pathways that are involved in Mn toxicity.

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2 Chapter 2: 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

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2.1 Abstract

Heavy metal and metalloid contaminations are among the most concerning types of pollutant in the environment. Consequently, it is important to investigate the molecular mechanisms of cellular responses and detoxification pathways for these compounds in living organisms. To date, a number of genes have been linked to the detoxification process. The expression of these genes can be controlled at both transcriptional and translational levels. In baker's yeast, *Saccharomyces cerevisiae*, resistance to a wide range of toxic metals is regulated by glutathione S-transferases. Yeast *URE2* encodes for a protein that has glutathione peroxidase activity and is homologous to mammalian glutathione S-transferases. The *URE2* expression is critical to cell survival under heavy metal stress. Here, we report on the finding of two genes, *ITT1*, an inhibitor of translation termination, and *RPS1A*, a small ribosomal protein, that when deleted yeast cells exhibit similar metal sensitivity phenotypes to gene deletion strain for *URE2*. Neither of these genes were previously linked to metal toxicity. Our gene expression analysis illustrates that these two genes affect *URE2* mRNA expression at the level of translation.

2.2 Introduction

Heavy metals and metalloids comprise a group of elements that are loosely defined as relatively high-density transition metals and metalloids (Ferguson, 1990; Tamás and Martinoia, 2006). Different metals are found in varied concentrations across the environment. Some of these heavy elements, such as iron (Fe), cobalt (Co) and zinc (Zn), are essential nutrients, while others are relatively harmless at low concentrations such as rubidium (Rb), silver (Ag) and indium (In). At higher concentrations, all metals and metalloids derived from natural environment (Goyer and Clarkson, 1996) or anthropogenic sources such as phosphate fertilizers, disinfectants, fungicides, sewage sludge, industrial waste, bad watering practices in agricultural lands, and dust from smelters (Schwartz *et al.*, 2001; Chronopoulos *et al.*, 1997) are toxic to living cells (Chronopoulos *et al.*, 1997; Violante *et al.*, 2010; Hernández *et al.*, 2015). Among these, arsenic (As) is one of the most toxic despite being the twentieth most abundant element on our planet. Its inorganic oxyanion forms including arsenite (As(III)) and arsenate (As(V)) are highly lethal to living organisms (Jaishankar *et al.*, 2014).

Over the course of evolution, many organisms have found ways, for example, by evolving molecular pathways to survive increased concentrations of metallic toxins in their environment (e.g. Basu *et al.*, 1997; Choudhury and Kumar, 1998; Castro-Silva *et al.*, 2003; Otth *et al.*, 2005). Microbes with extreme adaptation to heavy metals use detoxification pathways to reduce toxic metals to a lower redox state, which lessens their mobility and toxicity (Gadd, 2007). The baker's yeast, *Saccharomyces cerevisiae* possesses an effective mechanism to negate heavy metal and metalloids toxicity, allowing it to survive a broad range of toxic stress scenarios (Vadkertiová and Sláviková, 2006;

Hosiner *et al.*, 2014). This makes yeast an ideal model organism to study molecular mechanisms of the stress response that drive detoxification processes.

The glutathione S-transferases (GSTs) are key enzymes that mediate the resistance of *S. cerevisiae* to a wide range of heavy metals and metalloids. Yeast *Ureidosuccinate Transport 2 (URE2)* gene product is structurally homologous to mammalian GST and is a major player in the detoxification of *S. cerevisiae* against toxic metals through its glutathione peroxidase activity (Umland *et al.*, 2001; Bai *et al.*, 2004). For detoxification purposes, GST proteins catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates (Todorova *et al.*, 2010). The deletion strain for *URE2* is hypersensitive to a wide range of heavy metals and metalloids including As, Cd and nickel (Ni) (Todorova *et al.*, 2010; Rai *et al.*, 2003). In this report, we show that the deletion of either *ITTI* (inhibitor of translation termination 1) or *RPS1A* (small ribosomal subunit protein 10), makes the cells more sensitive to As(III), cadmium (Cd) and Ni suggesting a functional connection of these two genes with heavy metal toxicity. Itt1p is known to modulate the efficiency of translation termination through physical interactions with two eukaryotic release factors eRF1 (Sup45p) and eRF3 (Sup35p) (Urakov *et al.*, 2001). Rps1Ap is a constituent of the small ribosomal subunit; little information is known about its function in *S. cerevisiae* (Swoboda *et al.*, 1995). Neither of these genes had previously been linked to heavy metal toxicity. Overall, we provide evidence that the connection for *ITTI* and *RPS1A* with heavy metal toxicity is through their influence on the translation of *URE2* gene.

2.3 Materials and methods

2.3.1 Strains and plasmids used in this study

Yeast, *S. cerevisiae*, mating type (a) MATa strain BY4741 (*MATa orf4::KanMAX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and mating type (α) MATa strain, BY7092 (*MATa can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu21Δ0 ura3Δ0 met15Δ0*) (Tong *et al.*, 2001) were utilized for this study. The yeast MATa knockout (YKO) collection (Tong *et al.*, 2001) and PCR-based transformed cells were used as a source of gene deletion mutants; the open reading frame (ORF) collection (Sopko *et al.*, 2006) was used for over expression plasmid vectors. Yeast GFP Clones (Huh *et al.*, 2003) were modified for Western blot analysis. pAG25 plasmid containing the Nourseothricin Sulfate (clonNAT) resistance gene was used as a DNA template in PCR to generate gene knockouts. *Escherichia coli* strain DH5α was used to replicate different plasmids (Taylor *et al.*, 1993). Two plasmids were used carrying the *β-galactosidase* open reading frame for quantification of *URE2*-IRES and cap-dependent translation activities. p281-4-*URE2* contained a *URE2*-IRES region which was fused with *LacZ* (*β-galactosidase*) gene from *E. coli*, and p281 contained only the *β-galactosidase* gene as a control for cap-dependent translation (Komar *et al.*, 2003). All plasmids carried an ampicillin resistance gene which was used as selectable marker in *E. coli* and the *URAcil requiring 3* (*URA3*) marker gene which was used for selection in yeast.

2.3.2 Media and MiniPrep

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 cells were grown at 30°C unless otherwise indicated in the SGA procedure. *E. coli* cells were grown at 37°C. Yeast plasmid extraction was performed by using yeast plasmid mini kit (Omega Bio-tek®) and *E. coli* plasmid extraction was carried out by using GeneJET plasmid miniprep kit (Thermofisher®) according to the manufacturer's instructions.

2.3.3 Gene knockout and DNA transformation

Mutant strains were either selected from the library of gene deletions (Tong *et al.*, 2001) in MATa haploid form or a PCR-based gene knockout approach was used to achieve gene deletion. Targeted gene knockout strains were generated by PCR-based gene deletion strategy utilizing the clonNAT selection gene (Omidi *et al.*, 2018; Omidi *et al.*, 2014). Plasmid and gene transformation were performed by using a chemical-transformation strategy (LiOAc method) and confirmed via colony PCR (Alamgir *et al.*, 2010; Samanfar *et al.*, 2017).

2.3.4 Chemical sensitivity

Colony count assay (spot test) was performed to estimate the cell sensitivities, based on their ability to give rise to colonies. Strains were grown in liquid YPD or SC without uracil to saturation phase and serially diluted in sterile distilled H₂O to 10⁻⁴ and aliquots were streaked on solid media. The cells were cultured on YP, and YP supplemented with Na₃AsO₃ (As(III) (1 mM)), CdCl₂ (Cd (0.1 mM)), NiCl₂ (Ni (8 mM)), as well as 6% ethanol and 6% ethanol + Na₃AsO₃ (As(III) (0.3 mM)) for two days at 30°C. YP + 2% glucose (YPD) was used for the experiments without the involvement of

any plasmid and YP + 2% galactose (YPG) was used for experiments harboring plasmids (overexpression) with a galactose-inducible promoter (GAL1/10), in order to activate the desired gene. The number of colonies under drug conditions were compared to the number of colonies formed under non-drug conditions for consistency and subsequently normalized to the number of colonies formed by wild type (WT) strain under the same condition. Each experiment was repeated at least three times.

2.3.5 Quantitative β -galactosidase assay

ortho-Nitrophenyl- β -galactoside (ONPG)-based β -galactosidase analysis was used to quantify involvement of the identified genes in the IRES-mediated translation of *URE2* via quantification of β -galactosidase activity produced by a plasmid containing *URE2*-IRES fused to a β -galactosidase reporter (p281-4-*URE2*) (Komar *et al.*, 2003). This plasmid contains a DNA sequence that forms four strong hairpin loops prior to *URE2*-IRES region at its mRNA level. These four hairpin loops inhibit cap-dependent translation of *URE2* mRNA. A background plasmid (p281) carrying only a β -galactosidase reporter was used as a control for cap-dependent translation (Komar *et al.*, 2003). Both plasmids contained a GAL1/10 promoter and YPG was used as a medium to activate the desired gene (Samanfar *et al.*, 2014). Each experiment was repeated at least three times.

2.3.6 Reverse Transcriptase quantitative PCR (RT-qPCR)

This methodology was used to assess the content of target mRNAs. Total mRNA was reverse-transcribed into complementary DNA (cDNA), 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). Total RNA extractions were performed with Qiagen RNeasy Mini Kit (Qiagen®). 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. In this experiment, *PGK1* was used as a constitutive housekeeping gene and related to WT (Samanfar *et al.*, 2017; 2014). Each qPCR experiment was repeated at least three times using separate cDNA samples. Following oligonucleotide primers were used to quantify *URE2* and *PGK1* mRNAs:

Forward *URE2*: ATGATGAATAACAACGGCAA

Revers *URE2*: TCATTCACCACGCAATGCCT

Forward *PGK1*: ATGTCTTTATCTTCAAAGTT

Revers *PGK1*: TTATTTCTTTTCGGATAAGA

2.3.7 Immunoblotting

Western blotting was used to quantify relative protein levels. Total protein was isolated using detergent-free methods as described in (Szymanski and Kerscher., 2013). Samples were grown overnight in liquid YPD, pelleted and washed with PBS buffer. Samples for As(III) treatment, were grown overnight in liquid YPD and then treated with Na₃AsO₃ (As(III) (0.5 mM)) for 2 hours. Bicinchoninic acid assay (BCA) (Thermofisher®) was used to quantify total protein concentration according to manufacturer's instructions. 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®) (Shaikho *et al.*, 2016). Proteins were transferred to a nitrocellulose 0.45 µm paper (Bio-Rad®) via a Trans-Blot Semi-Dry Transfer (Bio-Rad®). Mouse

monoclonal anti-GFP antibody (Santa Cruz®) was used to detect protein level of Ure2p in Ure2-GFP protein fusion strains. Pgk1p was also used as a constitutive housekeeping protein for quantification purposes. Mouse anti-Pgk1 (Abcam®) was used to detect Pgk1p levels (Samanfar *et al.*, 2017). 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®), and each density was normalized to the density formed by Pgk1p control. Each experiment was repeated at least three times using three separate total protein isolates.

2.3.8 Polyribosome fractionation

The total RNA extraction from yeast cells was adapted from (Esposito and Mateyak, 2010). Overnight cell culture was used to inoculate YPD liquid medium. Prior to extraction, cycloheximide (100 µg/ml) was added to the samples for 15 minutes. Yeast culture was harvested at mid-log phase (OD₆₀₀ 0.6–0.8) and immediately chilled on dry ice prior to lysing the cells. Yeast cells were lysed by mechanical disruption using 425 – 600 µm acid-washed glass beads (Sigma®). Fifty µg of the total RNA was then loaded on a 10–50% sucrose gradient (20 mM Tris pH 8, 140 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 100 µg/ml cycloheximide and sucrose to desired concentration). An Automated Gradient Maker (Biocomp gradient maker) was used to produce the sucrose gradients. Centrifugation was performed at 40,000 rpm for 2 hours at 4°C (Beckman Optima LE-80K Ultracentrifuge) to separate the particles according to relative sedimentation rate.

Samples were analysed via a Biocomp Gradient Station immediately after

centrifugation. The instrument recorded A_{254} using a flow cell coupled with a spectrophotometer (Bio-Rad Econo UV monitor). In this procedure, untranslated mRNAs (top fractions) are separated from polysome-associated mRNAs (bottom fractions) as described in (Faye *et al.*, 2014). Fractions were collected (~650 μ l) using Bio-Rad Collection Station (Bio-Rad®) and adjusted to 1% SDS for fluctuation analysis of *URE2* mRNA level via RT-qPCR (Lou *et al.*, 2014). Each polysome profiling experiment was repeated at least three times.

Luciferase RNA (0.1 μ g/ml) (Promega®) was then added to each fraction as a control. RNA was precipitated overnight and purified as described in (Lou *et al.*, 2014), by using Glycoblue and acidic Phenol/chloroform (pH 4). Purified RNA samples were subjected to quantification for *URE2* mRNA by performing RT-qPCR as described in the above section. The normalized values for each fraction was determined by using the C_t values for *URE2* and the C_t values for the gene of interest using the formula $[2^{(C_t \text{ luciferase} - C_t \text{ target gene})}]$ (Sehgal *et al.*, 2008). The relative amount of *URE2* mRNA was calculated by dividing the amount in each fraction by the total signal in all fractions (Sehgal *et al.*, 2008; Chassé *et al.*, 2016). RT-qPCR analysis for polysome profiling was repeated at least three times using fractions from three separate polysome profiles.

2.3.9 Genetic interaction (GI) and conditional GI analysis

Synthetic genetic array (SGA) analysis was performed in large-scale through the creation of double-mutations and subsequent analysis of colony size (fitness) as previously described in (Samanfar *et al.*, 2017; Tong *et al.*, 2004). In summary, both gene candidates *ITTI* and *RPS1A* were knocked out in MAT α (BY7092) and crossed with two

arrays of haploid MATa knock-out strains (Samanfar *et al.*, 2017). The first array contained 384 deletion strains for genes that are directly or indirectly involved in the process of translation (translation gene deletion array). The second contained 384 random genes that were selected from the YKO collection (Tong *et al.*, 2001; Samanfar *et al.*, 2017), which was used as a control. Selectable markers designated in each background mating type, allowed for multiple selection steps. Meiotic progeny harboring both mutations were selected. The created arrays could then be used to score double mutants for their altered fitness under certain conditions (Tong *et al.*, 2001).

Colony size of both single mutant arrays (reference and control) and double mutant arrays were measured for their colony fitness (Memarian *et al.*, 2007; (Wagih *et al.*, 2013). After three repeats, the interactions with 20% alteration or more in at least two repeats were considered positive hits. Conditional SGA analysis was carried on under sub-inhibitory concentrations of chemicals (0.7 mM for As(III) and 60 ng/ml for cycloheximide). Phenotypic suppression array (PSA) analysis infers closer functional relationships between interacting genes by establishing a more direct form of GI. It refers to situations where defective growth caused by deletion of a particular gene, in a specific growth condition (for example presence of a drug) is rescued by the overexpression of another. PSA analysis was performed under a high sub-inhibitory targeted condition (As(III) (1.2 mM) and cycloheximide (100 ng/ml)) as previously described in (Tong and Boone, 2007; Samanfar *et al.*, 2017), by overexpressing either *ITT1* or *RPS1A* in the above described translation gene deletion array. Each experiment was repeated three times and the interactions with 25% alteration or more in at least two screens were scored as positive.

2.4 Results and discussion

2.4.1 Deletion of *ITT1* or *RPS1A* increases yeast sensitivity to heavy metals

Understanding the biology of the stress that heavy metals exert on a cell, as well as the cellular responses and mechanisms that a cell uses for detoxification of these toxins, has been the subject of numerous investigations over the past decades (e.g. Tamás and Martinoia, 2006; Gadd, 2007; Vadkertiová and Sláviková, 2006; Hosiner *et al.*, 2014). Although much has been learned, additional studies are needed to uncover details of such responses as well as additional genes that may participate in this process. To this end, while screening for yeast gene deletion mutants against heavy metals, we identified two deletion mutant strains for *ITT1* and *RPS1A* that showed increased sensitivity to three heavy metals (As, Ni and Cd). These two genes are part of a larger investigation to study the chemical-genetic profile of heavy metals in yeast (e.g. Márquez *et al.*, 2018); details of this study will be published elsewhere. In our spot test sensitivity analysis, when As(III) (1 mM), Cd (0.1 mM) and Ni (8 mM) were added to the solid media the number of normalized yeast colony counts were significantly reduced for *itt1Δ* and *rps1aΔ* strains (Fig 2-1A), highlighting the sensitivity of the mutant strains to these metals and metalloids. Rescue experiments revealed a complete recovery to heavy metal toxicity upon the reintroduction of the deleted genes into their corresponding mutants (Fig 2-1B). This reversion of sensitivity indicates that the sensitivity phenotypes are in fact a consequence of the intended gene deletions and are not due to a possible secondary mutation within the genome. The sensitivity of deletion mutants to As, Cd and Ni suggests a potential association for the target genes to heavy metal toxicity, a unique

observation that has not been previously reported.

Since the function of both *ITT1* and *RPS1A* can be linked to the process of protein biosynthesis (Urakov *et al.*, 2001; Swoboda *et al.*, 1995), it is conceivable that these genes may indirectly influence heavy metal sensitivity by regulating the activity of another gene. However, given that the Ure2p is reported to be a key enzyme involved in heavy metal detoxification in yeast (Todorova *et al.*, 2010; Rai *et al.*, 2003), we made double gene deletion mutants for *ITT1* and *RPS1A* with *URE2* and exposed them to As(III) (1 mM) for further analysis (Fig 2-1C). The sensitivity analysis of the double gene deletion mutants indicated no increased sensitivity to As(III) in addition to that observed for the single gene deletion mutant for *URE2*. This specifies a dominant effect for *URE2* on heavy metal sensitivity over *ITT1* and *RPS1A* (Fig 2-1C). To further support the observed phenotype, we introduced plasmid vectors containing *ITT1* and *RPS1A* into the deletion strain of *URE2* (Fig 2-1D). The results demonstrated the same levels of sensitivity to As(III) with no compensation, deeming indirect roles of *ITT1* and *RPS1A* in rescuing the cells from As(III) toxicity when *URE2* is deleted. One way to explain this data is that *ITT1* and *RPS1A* may exert their effect on sensitivity via the same pathway as *URE2*. If *ITT1* and *RPS1A* influenced a second pathway, it might be expected that their deletion would have had an additional effect on sensitivity when combined with *URE2* deletion (Márquez *et al.*, 2018). However, this was not observed. On the other hand, overexpression of *URE2* in *itt1Δ* and *rps1aΔ* strains reversed the As(III) sensitivity phenotype observed by the corresponding gene deletions, effectively deeming these gene deletions inconsequential for heavy metal sensitivity (Fig 2-1E). These observations are in accordance with the activity of *URE2* as a dominant player in heavy metal toxicity and

that it functions downstream of *ITT1* and *RPS1A*. Finally, to ensure the specific sensitivity of *itt1Δ* and *rps1aΔ* strains to heavy metals, we investigated the introduction of different oxidative stress conditions such as methyl methanesulfonate (MMS), acetic acid, and heat shock (Appendix 2-1 Fig 2-S1).

Figure 2-1: Normalized CFU counts for different yeast strains after exposure to As(III) (1 mM). CFU counts after 3 days exposure to the experimental condition are normalized to control condition counts. (A) Sensitivity of *itt1Δ*, *rps1aΔ* and *ure2Δ* compare to WT phenotype. (B) Rescued sensitivity of all deletion strains by reintroduction of their overexpression plasmids. (C) Sensitivity analysis for double gene deletions for *ITT1* or *RPS1A* in the absence of *URE2* compared to single gene deletion of *URE2*. (D) Sensitivity analysis for overexpression of *ITT1* or *RPS1A* in the absence of *URE2*. (E) sensitivity of *itt1Δ*, *rps1aΔ* after introduction of pURE2 (carries *URE2* genes). Each experiment was repeated at least three times. Error bars are calculated as standard deviations. * Indicates statistically significant differences (t-test) between WT cells and mutant cells treated with heavy metals (p < 0.005). Colour code is the same as in (A) for all panels.

Our findings suggest that the influence of *ITT1* and *RPS1A* on heavy metal sensitivity is linked to *URE2*. Although both *ITT1* and *RPS1A* have reported roles in protein biosynthesis (Urakov *et al.*, 2001; Swoboda *et al.*, 1995), the possible mechanism, regulation of transcription or translation of *URE2* remains to be investigated.

2.4.2 *ITT1* and *RPS1A* do not affect the expression of *URE2* at the mRNA level

RT-qPCR was employed to detect possible changes to *URE2* mRNA levels in the absence of *ITT1* and *RPS1A*. Figure 2-2 illustrates our observation that in comparison to WT, the *URE2* mRNA appeared unchanged in the mutant strains, *itt1Δ* and *rps1aΔ*. We also investigated the content of *URE2* mRNA after exposure to As(III) (0.5 mM). As noted before, neither the deletion of *ITT1* or *RPS1A* appeared to influence the content of *URE2* mRNA, suggesting that the activity of *ITT1* and *RPS1A* is independent of *URE2* mRNA content.

Figure 2-2: The relative *URE2* mRNA level quantified by normalizing the mRNA content of the mutant strains to those in the wild type. The house keeping gene *PGK1* remained constant under both conditions and was used as an internal control to normalize the mRNA levels of *URE2* mRNA in the absence of *ITT1* and *RPS1A* to WT. Deletion of *ITT1* or *RPS1A* had no effect on the normalized *URE2* mRNA content. Each experiment was repeated at least three times. Error bars represent standard deviations.

2.4.3 Ure2p content is reduced in the absence of *ITT1* or *RPS1A*

We investigated the level of Ure2p in the presence and absence of *ITT1* or *RPS1A* in the cells by Western blot analysis. This was accomplished using a strain that carried Ure2p fused to a GFP at the genomic level. Our analyses show a reduction in the endogenously-expressed Ure2-GFP fusion protein levels in the absence of *ITT1* or *RPS1A* (Fig 2-3). When either *ITT1* or *RPS1A* were deleted, Ure2p levels were reduced by approximately 40% and 60%, respectively, compared to the WT cells (Fig 2-3A). This suggests that *ITT1* and *RPS1A* play an imperative role in regulating the expression of Ure2p. In parallel, introduction of As(III) (0.5 mM) to the growth media reduced Ure2p approximately 70% and 80% for *Δitt1* and *Δrps1a*, respectively compared to WT strain (Fig 2-3B) which is reduced by approximately 34% in the presence of As (III) (0.5 mM) compared to normal experimental condition. Deletion of *ITT1* or *RPS1A* did not change the protein levels of Pgk1p, used as an internal control. These observations provide evidence by connecting *ITT1* and *RPS1A* activities to the level of Ure2p. The additional reduction in the Ure2p levels in the presence of As(III) implicates that *ITT1* and *RPS1A* may have a higher influence in regulation of Ure2p expression under a stress condition.

Figure 2-3: Western blot followed by densitometry analysis to measure Ure2-GFP levels in different yeast strains. Values are normalized to that for Pgk1p, used as an internal control and related to the values for WT strain. (A) Cells are grown under standard laboratory conditions. (B) Cells are challenged by As(III) (0.5 mM). In WT strain, normalized Ure2-GFP level was reduced by $34\% \pm 4\%$ in the presence of As(III) (0.5 mM). Each experiment was repeated at least three times. Error bars represent standard deviations. * Indicates statistically significant differences (t-test) between WT cells and mutant cells untreated and treated with As(III) ($p < 0.005$).

2.4.4 Analysis of the *URE2* translation

Since our data suggests a role for *ITT1* and *RPS1A* in modulating *URE2* expression at the protein level, polyribosome-bound mRNA analysis was performed. In this method, fractions of polysomes are isolated and analyzed for their content of a target mRNA. Those mRNAs that are translated more efficiently are generally found in association with multiple ribosomes and hence will be isolated in heavier polysome fractions. In contrast, those mRNAs, which are translated to a lesser degree, can be found in lower density fractions (Shaikho *et al.*, 2016). In this way, the distribution of mRNAs within polysome fractions can be used to estimate the translation efficiency of the target mRNA. Using this strategy, polysome profile analysis was performed for *URE2* mRNA,

in the presence or absence of *ITT1* and *RPS1A*. Analysis of the polysome fractions for *URE2* mRNA content using RT-qPCR, normalized to a control (housekeeping) mRNA (*PGK1*), showed a shift in *URE2* mRNA accumulation towards lighter polysome fractions for mutant strains (Fig 2-4), suggesting that when *ITT1* or *RPS1A* are deleted, *URE2* mRNA is translated to a less efficiently. These data provide direct evidence that *ITT1* and *RPS1A* affect the translation of *URE2* mRNA.

Figure 2-4: Polysome-bound mRNA analysis of *itt1Δ*, *rps1aΔ* and WT strains. The amount of *URE2* mRNA in each fraction was determined by RT-qPCR and the percentage of total *URE2* mRNA on the gradient is plotted for each fraction. The profiles of *PGK1* mRNA, used as an internal control, were similar for deletion and WT strains and were used to normalize other values. Each experiment was repeated at least three times. Error bars represent standard deviations.

2.4.5 Ethanol increases As(III) sensitivity for *ITT1* and *RPS1A* gene deletion strains

In addition to cap-dependent translation, *URE2* mRNA has been shown to undergo a cap-independent translation, which represents an interesting mode of gene

expression control (Komar *et al.*, 2003). Cap-dependent translation is mediated through the scanning of mRNA 5' UTR to find a suitable start codon. In cap-independent translation, mRNA structures known as IRES, mediate the interaction between ribosomes and the mRNA, independently of the 5'cap (Holcik and Sonenberg, 2005). IRES-mediated translation is mainly used by RNA viruses, but it can also be found in cellular mRNAs (Pelletier and Sonenberg, 1988; Liwak *et al.*, 2012). The majority of translation in eukaryotes occurs through cap-dependent translation, whereas IRES-mediated translation is often associated with physiological conditions such as stress, where general translation is compromised (Holcik and Sonenberg, 2005; Stoneley and Willis, 2004; Komar and Hatzoglou, 2011). Knowing that the exposure to heavy metal causes a stress condition for yeast cells (e.g. Todorova *et al.*, 2010; Spriggs *et al.*, 2008) we examined the possibility that *ITT1* and *RPS1A* may influence IRES-mediated *URE2* mRNA translation. As a quick measure to examine this possibility, ethanol sensitivity analysis was used as a positive stress control. Eukaryotic initiation factor 2A (eIF2A) acts as a regulator of IRES-mediated translation in *S. cerevisiae* cells (Reineke *et al.*, 2011). Abundance of eIF2A is shown to specifically repress IRES-mediated translation of *URE2* as well as other yeast genes with IRES forming region (Reineke *et al.*, 2011). It was previously reported that ethanol reduces the expression of eIF2A at protein levels and hence promotes translation via IRES elements (Reineke *et al.*, 2011).

As described, *URE2* plays a critical role in heavy metal detoxification (Komar and Hatzoglou, 2011; Todorova *et al.*, 2010). If *ITT1* and *RPS1A* affect IRES-mediated translation of *URE2* mRNA, one may expect the presence of ethanol to further enhance the sensitivity of *itt1Δ* and *rps1aΔ* to As(III) treatment. In this case, presence of ethanol

and deletion of either *ITT1* or *RPS1A* could be considered to introduce a double effect on the same overall process.

As demonstrated in Figure 2-5, *ure2Δ*, *itt1Δ* and *rps1aΔ* showed hypersensitivity to a very low concentration of As(III) (0.3 mM) in the presence of 6% ethanol which suggests that ethanol increases the sensitivity of mutant strains to As(III). The mutant strains did not exhibit sensitivity to neither 0.3 mM of As(III) nor 6% ethanol separately. These data may suggest a connection for the activities of *ITT1* and *RPS1A* to IRES-mediated translation. These outcomes are open to other explanations, for example, ethanol and As(III) may have alternative effects on the cell independent of IRES-mediated translation and hence influence cell viability. In this context double deletion strains for eIF2A with *ITT1* or *RPS1A* showed similar sensitivity to the presence of either As(III) (0.3 mM) alone or with ethanol (6%) (Fig 2-5) connecting the reported activity of ethanol to eIF2A. Nonetheless, additional experiments are needed to confirm these interpretations. A double gene deletion for *ITT1* or *RPS1A* with *URE2* did not result in additional sensitivity compared to that observed for *URE2* single gene deletion. This additionally reiterates the connection between the activities of *ITT1* and *RPS1A* via *URE2* expression.

Figure 2-5: Average viability of single and double gene deletion strains for *ITT1* and *RPS1A* with *URE2*. Cells were exposed to As(III) (0.3 mM), 6% ethanol, a combination of both (6% ethanol + As(III) (0.3 mM)) or no treatment (control). CFU counts after 3 days of exposure to the experimental conditions are normalized to CFU counts for WT strain. Error bars represent standard deviation of at least three independent experiments. * Indicates statistically significant differences (t-test) between WT cells and mutant cells treated with As(III) + ethanol (p < 0.005).

2.4.6 *ITT1* and *RPS1A* affect IRES-mediated translation of a β -galactosidase reporter gene

To further study the effect of *ITT1* and *RPS1A* on IRES-mediated translation of *URE2*, we used a β -galactosidase reporter whereby the β -gal mRNA is under the translational control of the *URE2*-IRES element. For this purpose, we utilized a previously published plasmid construct known as p281-4-*URE2* (Fig 2-6) (Komar *et al.*, 2003; Reineke *et al.*, 2011).

Figure 2-6: Effect of gene deletion on translation and transcription of β -galactosidase mRNA. (A) The relative β -galactosidase activity is determined by normalizing the activity of the mutant strains to that of the WT strain. Blue bars represent β -galactosidase activity under the translational control of *URE2-IRES*. Red bars represent β -galactosidase activity via cap-dependent translation. (B) The relative β -galactosidase mRNA level quantified by normalizing the mRNA content of the mutant strains to those in the wild type. The house keeping gene *PGK1* was used as an internal control to normalize the mRNA levels of β -galactosidase mRNA in WT, *itt1* Δ and *rps1a* Δ . Deletion of *ITT1* or *RPS1A* had no effect on the normalized β -galactosidase mRNA content. Each experiment was repeated at least three times. Error bars represent standard deviations. * Indicates statistically significant differences (t-test) between WT cells and mutant cells ($p < 0.005$).

As shown in Figure 2-6, deletion of either *ITT1* or *RPS1A* resulted in reduced expression of β -galactosidase under the translational control of *URE2-IRES*. This data further supports the notion that *ITT1* and *RPS1A* regulate *URE2-IRES*-mediated translation. As a control to account for cap-dependent translation activity, p281 background construct (Komar *et al.*, 2003; Reineke *et al.*, 2011) carrying a β -galactosidase mRNA lacking four hairpin loops and *URE2-IRES* was utilized. We observed no significant difference in β -galactosidase activity for WT, $\Delta itt1$ and $\Delta rps1a$ strains indicating that *ITT1* and *RPS1A* do not influence cap-dependent translation (Fig 2-6). Since the reduced levels of β -galactosidase may be a result of reduced mRNA, the β -galactosidase mRNA was evaluated. As expected, we observed no significant change in its mRNA level in the absence of neither *ITT1* nor *RPS1A* (Fig 2-6B).

2.4.7 Genetic interaction analysis further connects the activity of *ITT1* and *RPS1A* to regulation of translation in response to stress

To further examine the role of *ITT1* and *RPS1A* in the process of translation, we studied the genetic interactions (GIs) by screening *ITT1* and *RPS1A* against an array of 384 genes associated with protein biosynthesis and a second set of 384 random genes used for control purposes. Genes that are functionally related often partake in GIs (also known as epistatic interactions) (Rieger *et al.*, 2012; Szendro *et al.*, 2013). The most commonly studied form of GI is known as negative GI, where the reduced fitness or lethal phenotype of a double mutant strain for missing two genes is not observed in single mutant strains (Samanfar *et al.*, 2017). Genes that are associated with parallel and compensating pathways are thought to commonly form negative GIs (Tong *et al.*, 2004; Samanfar *et al.*, 2017). Under standard laboratory growth conditions, both *ITT1* and *RPS1A* exhibited negative GIs with a limited number of genes involved in the process of protein biosynthesis (Figs 2-7A and B). This is expected as both genes are thought to be involved in the process of translation. Examples of genes that formed GIs with *ITT1* and *RPS1A* are large ribosomal subunit protein 19A (*RPL19A*) and general control non-derepressible (*GCN3*), respectively. Rpl19p is a conserved large ribosomal subunit protein involved in ribosomal intersubunit bridging and its alteration is connected to the fidelity of translation (Kisly *et al.*, 2016; VanNice *et al.*, 2016). On the other hand, *GCN3* is the alpha subunit of translation initiation factor 2B (eIF2B), which is involved in guanine-nucleotide exchange for eIF2. In this manner, phosphorylation of eIF2B regulates the activity of Gcn3p (Hannig and Hinnebusch, 1988; Elsby *et al.*, 2011)

Conditional GIs define an interesting type of GIs as they provide more compelling

insight on the function of target genes under specific conditions (Babu *et al.*, 2011; Samanfar *et al.*, 2017). They represent the mosaic nature of gene function(s) which can change as a result of different external or internal factors. For example, although a number of genes are known to play a role in the DNA repair pathway, their expression is only regulated in response to the presence of DNA damage (Tong and Boone, 2007; Omid *et al.*, 2014). We therefore investigated the negative GIs by assaying *ITT1* and *RPS1A* in the presence of a low concentration of As(III) (0.7 mM). Under this condition, we found both genes to form a new set of interactions with a series of genes that play a role in the regulation of translation (Figs 2-7A and B). This data suggests that in the presence of As(III), both genes appear to gain a new role in regulating the process of translation (p-value *ITT1* = 9.39e-5 and p-value *RPS1A* = 5.26e-10) (Figs 7A and B). Suppressor of ToM1 (*STMI*) is an example of a gene that formed new negative genetic interactions in the presence of As(III) with both *ITT1* and *RPS1A*. *STMI* codes for a protein that is required for optimal translation under nutrient stress (Utsugi *et al.*, 1995; Nelson *et al.*, 2000). Enhancer of mRNA DeCapping *EDC1* and its paralog *EDC2* are other examples of the negative interactions gained by *RPS1A* under As(III) condition. Edc proteins directly bind to mRNA substrates and activate mRNA decapping. They also play a role in translation during stress conditions such as heat shock (Dunckle *et al.*, 2001). We also studied negative GIs in the presence of cycloheximide, which binds to the E-site of the 60S ribosomal subunit and interferes with deacylated tRNA to inhibit general protein synthesis in the cell (Schneider-Poetsch *et al.*, 2010) (Figs 2-7A and B). Similar to our observations with As(III), in the presence of a mild concentration of cycloheximide (60 ng/ml), *ITT1* and *RPS1A* formed new negative GIs with a group of genes that are

associated with the regulation of translation (p-value *ITT1* = 1.33e-6 and p-value *RPS1A* = 6.59e-9). Translation Initiation Factor 4A (*TIF2*) is an example of the gained interaction for both *ITT1* and *RPS1A* in response to cycloheximide. *TIF2* is a key player in translation initiation and holds a helicase activity (Linder and Slonimski, 1989). Altogether, these observations are in agreement with the involvement of *ITT1* and *RPS1A* in regulating translation in response to stress.

Figure 2-7: Genetic interaction (GI) analysis for *ITT1* and *RPS1A*. (A) Negative GIs for *ITT1* under standard growth conditions and the presence of As(III) or cycloheximide. In this case, deletion of a second gene along with *ITT1* forms an unexpected growth reduction. (B) Negative GIs for *RPS1A* under standard growth conditions and the presence of As(III) or cycloheximide as in (A). (C) Phenotypic suppression array (PSA) analysis using the overexpression of *ITT1*. In this way, overexpression for *ITT1* compensated for the sensitivity of gene deletions to As(III) (1.2 mM) or cyclohexamide (100 ng/ml). (D) PSA analysis using the overexpression of *RPS1A* as in (C). Each experiment was repeated three times and the interactions with 20% alteration or more in at least two screens were scored as positive. P-values were obtained from GeneMANIA.

To further study *ITT1* and *RPS1A*, we conducted phenotypic suppression array (PSA) analysis (Alamgir *et al.*, 2008; Samanfar *et al.*, 2017) (Figs 2-7C and D) to assess the ability of overexpression of our target genes to reverse the defective phenotype (i.e. sensitivity) on a series of gene deletion strains under specific conditions. PSA analysis constitutes a more direct form of GI and can infer close functional relationships between interacting genes. In these cases, overexpression of one gene compensates the phenotypic adverse effect that is caused by the absence of another gene under certain conditions, such as stress caused by different chemicals (Alamgir *et al.*, 2010; Samanfar *et al.*, 2017). We observed that the overexpression of both *ITT1* and *RPS1A* reversed the sensitivity of a number of gene deletion strains to As(III) (1.2 mM) or cycloheximide (100 ng/ml) (Figs 2-7C and D). The majority of the newly identified gene interactors are involved in translation regulation further connecting the activity of *ITT1* and *RPS1A* to the regulation of translation. Interestingly, two of these genes, *GIS2* and *DOM34*, have reported to have IRES trans-acting factor (ITAF) activity Scherrer *et al.*, 2011; Sammons *et al.*, 2011; Samanfar *et al.*, 2017). The fact that the overexpression of *ITT1* and *RPS1A* can compensate for the absence of ITAFs, *GIS2* and *DOM34* provide further evidence connecting *ITT1* and *RPS1A* to IRES-mediated translation.

GIg Suppressor (*GIS2*) is a well-studied translational activator for numerous IRES containing mRNAs Scherrer *et al.*, 2011; Sammons *et al.*, 2011). Duplication Of Multilocus region 34 (*DOM34*) is a protein that facilitates inactive ribosomal subunit dissociation to aid in translation restart (Davis and Engebrecht, 1998), and is reported to play a role in IRES-mediated translation in yeast (Samanfar *et al.*, 2017). Our PSA analysis, not only supports a role in IRES-mediated translation pathway for both *ITT1* and

RPS1A, but also suggests a possible systematic compensation between certain ITAFs which can be the subject of future studies. This also leads to the conclusion, that other interacting partners of *ITTI* and *RPS1A* in this experiment may play a role in IRES-mediated translation. Further studies are required to investigate these hypotheses. In agreement with our findings here, Sammons *et al.*, in 2011 reported a physical interaction between Rpsa1p and Gis2p connecting the activity of these two proteins.

3 Chapter 3: Deleting *UGA4* and *YBR062C* increases heavy metal sensitivity in yeast, *Saccharomyces cerevisiae*

3.1 Abstract

Concerns about the toxicity of heavy metal and metalloid contamination in our environment are on the rise. Understanding the molecular mechanisms that mediate heavy metal detoxification is important to our efforts to remove them from the environment and discern the cellular responses of affected species. Several genes are identified to play central roles in cellular detoxification process and the expression of said genes can be influenced at both the transcriptional and translational level by the presence of heavy metals. Cadmium (Cd) is among the most toxic elements in regard to human health; cadmium compounds are classified as human carcinogens by several regulatory agencies around the world. Many microorganisms are known to have evolved specific molecular pathways to overcome the toxicity of heavy elements such as Cd. Among those, resistance of budding yeast (*Saccharomyces cerevisiae*) to a wide range of toxic metals is mediated by glutathione S-transferase products. Yeast Ure2p, which is homologous to mammalian glutathione S-transferases and has glutathione peroxidase activity, becomes an essential protein under heavy metal stress. To identify genes that coordinate with Ure2p to respond to heavy metal toxicity stress and better understand their role in detoxification, we found two genes, *UGA4* and *YBR062C*, whose corresponding knockout strains exhibit similar drug sensitivity phenotypes to the knockout strain for *URE2*. No connection to heavy metal toxicity has been reported for either of these genes but the mechanisms by which they confer resistance is of interest. In this study, our extensive gene expression analysis indicates that *UGA4* and *YBR062C* significantly affect the rate of translation of *URE2*. These novel gene functions help provide a more comprehensive understanding of the mechanisms that contribute to effective heavy-metal detoxification strategies at the cellular level.

3.2 Introduction

Heavy metals within the Earth's crust include essential elements such as Fe, Co and Zn and others which are relatively harmless at low concentrations such as Ru, Ag, and In. Only a small selection of heavy metals is highly toxic to all living organisms, even at very low concentrations. These highly-toxic heavy metals include cadmium (Cd) and nickel (Ni) which have been long-term hazards to human health (Lin, 1998; Godt *et al.*, 2006; Violante *et al.*, 2010). Majority of human exposure to these kinds of elements generally happens through contact with a polluted environment and/or product which is often due to occupational hazards (Järup, 2003; Carson, 2018; Moteshareie *et al.*, 2018). A general increase in anthropogenic activities including mining operations, waste disposal and industrial, agricultural, and pharmaceutical manufacturing has led to a greater risk of heavy metal exposure (Schwartz *et al.*, 2001; Passariello *et al.*, 2002). Overtime, the increase anthropogenic activities have resulted in the accumulation of heavy metal pollutants in our environment, leading to increasing concerns regarding ecological and global public health (Carson, 2018).

Heavy metal pollution can also result from geogenic and atmospheric natural activities, leading to the constant exposure of microorganisms to increased concentrations of heavy metals over millions of years (e.g. Basu *et al.*, 1997; Choudhury *et al.*, 1998; Castro *et al.*, 2003; Otth *et al.*, 2005;). Cd and Ni are not highly abundant elements in the Earth's crust at only around 0.000015% and 0.009% availability respectively, but an increased use in industrial production has raised concerns about their toxicity. In the recent decades, augmented demands for rechargeable Ni-Cd batteries have increased the

risk of human exposure for extraction and disposal. Additionally, cadmium sulfate and cadmium selenide are used as stabilizers for polyvinyl plastic and alloys in electroplating (Rahimzadeh *et al.*, 2017). In response to such environmental hazards, the exposed organisms evolved effective molecular mechanisms to combat heavy metal toxicity. The mechanisms that govern detoxification in microorganisms are the subject of intense study to systematically investigate the role of each component of detoxification pathways (Castro *et al.*, 2003; Otth *et al.*, 2005; Rahimzadeh *et al.*, 2017; Moteshareie *et al.*, 2018).

Extreme adaptation to heavy metal pollution is possible through the use of detoxification pathways such as glutathione-mediated detoxification (GMD) process to reduce toxic metals to a lower redox state, which lessens their mobility and toxicity (Gadd, 2007). Budding yeast, *Saccharomyces cerevisiae* utilizes effective mechanisms to negate heavy metal toxic effects, allowing it to survive a broad range of toxic stress conditions (Vadkertiová and Sláviková, 2006; Hosiner *et al.*, 2014). Yeast is also a simple organism to work with, it is amenable for global-genetic analyses and easily adaptable to laboratory conditions, making it an excellent model organism to study and understand the molecular mechanisms underlying stress survival as well as detoxification processes (Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018).

In most microorganisms including yeast, metal intake first initiates with a nonspecific binding of the metal to the cell membrane and then toxic metals cross into the cytoplasm (Janicka-Russak, 2008; Ayangbenro and Babalola, 2017). For example, toxic metals can bind to sulphhydryl groups in membrane and or cytoplasmic proteins which can cause misfolding or deactivation. In addition to the formation of free radicals and reactive

oxygen species, they can replace an essential element which can have significant deficiency effects (Hall, 2002). These multiple interactions trigger a cascade of biochemical reactions to induce detoxification (Ayangbenro, and Babalola, 2017). An important component of the detoxification process is glutathione (GSH), a tripeptide present in all living cells formed from cysteine, glutamate, and glycine. GSH acts as an antioxidant to neutralize free Reactive Oxygen Species (ROS) and effectively prevents cellular damage (Grant *et al.*, 1996; Kumar *et al.*, 2011). Glutathione S-transferase (GST) is a family member of key enzymes that mediate the resistance of living cells to a wide range of heavy metal toxicity. GSTs catalyze the conjugation of the reduced form of GSH to xenobiotic substrates (Todorova *et al.*, 2010). Yeast *Ureidosuccinate Transport 2* (*URE2*) gene product possesses GST activity and homologous to mammalian GSTs. Ure2p is a major player in detoxification of *S. cerevisiae* against toxic metals through its glutathione peroxidase activity. When *URE2* is mutated and loss-of-function takes place, yeast cells become hypersensitive to a wide range of heavy metals including Cd, As, and Ni (Todorova *et al.*, 2010; Rai *et al.*, 2003).

In this study, we illustrate that deletion of either *UGA4* (*Utilization of Gaba 4*) and *YBR062C* (*unknown function*) makes the yeast cells more sensitive to Cd, As and Ni, thus suggesting a connection between these genes and heavy metal toxicity. Uga4p is a well-studied protein that serves as a 4-aminobutyric acid (GABA) transporter. It is involved in the utilization of GABA as a nitrogen source (Uemura *et al.*, 2004). Ybr062cp is an uncharacterized protein with unknown function. DeVit *et al.*, in 2005 proposed that *YBR062C* may play a role in activation of the filamentous growth pathway. They also illustrated that Ybr062cp physically interacts with Gis2p, GIg Suppressor (*GIS2*) which is

a well-studied translational activator for multiple IRES containing mRNAs (Sammons *et al.*, 2011; Moteshreie *et al.*, 2018). Neither one of these genes had been linked to heavy metal toxicity in the past studies. We provide evidence that the connection for *UGA4* and *YBR062C* with heavy metal toxicity is through their influence on the expression of *URE2* mRNA. Where deletion of both *UGA4* and *YBR062C* individually reduces the expression of *URE2* gene induced by Cd exposure.

3.3 Materials and methods

3.3.1 Strains and plasmids used in this study

Yeast, *S. cerevisiae*, mating type (a) MATa strain BY4741 (*MATa orfΔ::KanMAX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and mating type (α) MATα strain, BY7092 (*MATα can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu21Δ0 ura3Δ0 met15Δ0*) (Tong *et al.*, 2001) were utilized for this study. The yeast MATa knockout (YKO) collection (Tong *et al.*, 2001) and PCR-based transformed cells were used as a source of gene deletion mutants; the open reading frame (ORF) collection (Sopko *et al.*, 2006) was used for over expression plasmid vectors. Yeast GFP Clones (Huh *et al.*, 2003) were modified for Western blot analysis. pAG25 plasmid containing the Nourseothricin Sulfate (clonNAT) resistance gene was used as a DNA template in PCR to generate gene knockouts. *Escherichia coli* strain *DH5α* was used to replicate different plasmids (Taylor *et al.*, 1993). Two plasmids were used carrying the β -galactosidase open reading frame for quantification of *URE2*-IRES and cap-dependent translation activities. p281-4-*URE2* contained the *URE2*-IRES region fused with *LacZ* (β -galactosidase) gene from *E. coli*, and p281 contained only the β -galactosidase gene as a control for cap-dependent

translation (Komar *et al.*, 2003). All plasmids carried an ampicillin resistance gene which was used as selectable marker in *E. coli* and the *URA* requiring 3 (*URA3*) marker gene which was used for selection in yeast.

3.3.2 Media and MiniPrep

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 cells were grown at 30°C unless otherwise indicated in the SGA procedure. *E. coli* cells were grown at 37°C. Yeast plasmid extraction was performed by using Yeast Plasmid Mini Kit (Omega Bio-tek®) and *E. coli* plasmid extraction was carried out by using GeneJET Plasmid Miniprep kit (Thermofisher®) according to the manufacturer's instructions.

3.3.3 Gene knockout and DNA transformation

Mutant strains were either selected from the library of gene deletions (Tong *et al.*, 2001) in MATa haploid form or a PCR-based gene knockout approach was used to achieve gene deletion. Targeted gene knockout strains were generated by PCR-based homologous recombination gene deletion strategy utilizing the clonNAT selection gene (Omidi *et al.*, 2014; 2018). Plasmid and gene transformation were performed by using a chemical-transformation strategy (LiOAc method) and confirmed via colony PCR (Alamgir *et al.*, 2010; Samanfar *et al.*, 2017).

3.3.4 Chemical sensitivity

Colony count assay (spot test) was performed to estimate the cell sensitivities, based on their ability to give rise to colonies. Strains were grown in liquid YPD or SC without uracil to saturation phase and serially diluted in sterile distilled H₂O to 10⁻⁴ and aliquots were streaked on solid media. The cells were cultured on YP, and YP supplemented with Na₃AsO₃ (As(III) (1 mM)), CdCl₂ (Cd (0.1 mM)), NiCl₂ (Ni (8 mM)), as well as 6% ethanol and 6% ethanol + Na₃AsO₃ (As(III) (0.3 mM)) for two days at 30°C. YP + 2% glucose (YPD) was used for the experiments without the involvement of any plasmid and YP + 2% galactose (YPG) was used for experiments harboring plasmids (overexpression) with a galactose-inducible promoter (GAL1/10), in order to activate the desired gene. The number of colonies under drug conditions were compared to the number of colonies formed under non-drug conditions for consistency and subsequently normalized to the number of colonies formed by wild type (WT) strain under the same condition. Each experiment was repeated at least three times.

3.3.5 Quantitative β -galactosidase assay

ortho-Nitrophenyl- β -galactoside (ONPG)-based β -galactosidase analysis was used to quantify involvement of the identified genes in the canonical and IRES-mediated translation of *URE2* via quantification of β -galactosidase activity produced by a plasmid containing *URE2*-IRES fused to a β -galactosidase reporter (p281-4-*URE2*) (Komar *et al.*, 2003). This plasmid contains a DNA sequence that forms four strong hairpin loops prior to *URE2*-IRES region at its mRNA level. These four hairpin loops inhibit cap-dependent translation of *URE2* mRNA. A background plasmid (p281) carrying only a β -galactosidase reporter was used as a control for cap-dependent translation (Komar *et al.*,

2003). Both plasmids contained a GAL1/10 promoter and YPG was used as a medium to activate the desired gene (Samanfar *et al.*, 2014). Each experiment was repeated at least three times.

3.3.5 Reverse Transcriptase quantitative PCR (RT-qPCR)

This methodology was used to assess the content of target mRNAs. Total mRNA was reverse-transcribed into complementary DNA (cDNA), 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). Total RNA extractions were performed with Qiagen RNeasy Mini Kit (Qiagen®). 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. In this experiment, *PGK1* was used as a constitutive housekeeping gene and related to WT (Samanfar *et al.*, 2014; 2017). Each qPCR experiment was repeated at least three times using separate cDNA samples. Following oligonucleotide primers were used to quantify *URE2* and *PGK1* mRNAs:

Forward *URE2*: ATGATGAATAACAACGGCAA

Revers *URE2*: TCATTCACCACGCAATGCCT

Forward *PGK1*: ATGTCTTTATCTTCAAAGTT

Revers *PGK1*: TTATTTCTTTTCGGATAAGA

3.3.6 Immunoblotting

Western blotting was used to quantify relative protein levels. Total protein was isolated using detergent-free methods as described in (Szymanski and Kerscher., 2013). Samples were grown overnight in liquid YPD, pelleted and washed with PBS buffer.

Samples for As(III) treatment, were grown overnight in liquid YPD and then treated with Na_3AsO_3 (As(III) (0.5 mM)) for 2 hours. Bicinchoninic acid assay (BCA) (ThermoFisher®) was used to quantify total protein concentration according to manufacturer's instructions. Equal amounts of total protein (50µg) extract were loaded onto a 10% SDS-PAGE gel, run on Mini-PROTEAN Tetra cell electrophoresis apparatus system (Bio-Rad®) (Shaikho *et al.*, 2016). Proteins were transferred to a nitrocellulose 0.45 µm paper (Bio-Rad®) via a Trans-Blot Semi-Dry Transfer (Bio-Rad®). Mouse monoclonal anti-GFP antibody (Santa Cruz®) was used to detect protein level of Ure2p in Ure2-GFP protein fusion strains. Pgk1p was also used as a constitutive housekeeping protein for quantification purposes. Mouse anti-Pgk1 (Abcam®) was used to detect Pgk1p levels (Samanfar *et al.*, 2017). 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®), and each density was normalized to the density formed by Pgk1p control. Each experiment was repeated at least three times using three separate total protein isolates.

3.3.7 Polyribosome fractionation

The total RNA extraction from yeast cells was adapted from (Esposito and Mateyak, 2010). Overnight cell culture was used to inoculate YPD liquid medium. Prior to extraction, cycloheximide (100 µg/ml) was added to the samples for 15 minutes. Yeast culture was harvested at mid-log phase (OD_{600} 0.6–0.8) and immediately flash-frozen on dry ice prior to lysing the cells. Yeast cells were lysed by mechanical disruption using 425 – 600 µm acid-washed glass beads (Sigma®). Fifty µg of the total RNA was then

loaded on a 10–50% sucrose gradient (20 mM Tris pH 8, 140 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 100 µg/ml cycloheximide and sucrose to desired concentration). An Automated Gradient Maker (Biocomp gradient maker) was used to produce the sucrose gradients. Centrifugation was performed at 40,000 rpm for 2 hours at 4°C (Beckman Optima LE-80K Ultracentrifuge) to separate the particles according to relative sedimentation rate.

Samples were analysed via a Biocomp Gradient Station immediately after centrifugation. The instrument recorded A₂₅₄ using a flow cell coupled with a spectrophotometer (Bio-Rad Econo UV monitor). In this procedure, untranslated mRNAs (top fractions) are separated from polysome-associated mRNAs (bottom fractions) as described in (Faye *et al.*, 2014). Fractions were collected (~650 µl) using Bio-Rad Collection Station (Bio-Rad®) and adjusted to 1% SDS for fluctuation analysis of *URE2* mRNA level via RT-qPCR (Lou *et al.*, 2014). Each polysome profiling experiment was repeated at least three times.

Luciferase RNA (0.1 µg/ml) (Promega®) was then added to each fraction as a control. RNA was precipitated overnight and purified as described in (Lou *et al.*, 2014), by using Glycoblue and acidic Phenol/chloroform (pH 4). Purified RNA samples were subjected to quantification for *URE2* mRNA by performing RT-qPCR as described in the above section. The normalized values for each fraction was determined by using the Ct values for *URE2* and the Ct values for the gene of interest using the formula $[2^{-(Ct_{\text{luciferase}} - Ct_{\text{target gene}})]$ (Sehgal *et al.*, 2008). The relative amount of *URE2* mRNA was calculated by dividing the amount in each fraction by the total signal in all fractions (Sehgal *et al.*,

2008; Chassé *et al.*, 2016). RT-qPCR analysis for polysome profiling was repeated at least three times using fractions from three separate polysome profiles.

3.3.8 Genetic interaction (GI) and conditional GI analysis

Synthetic genetic array (SGA) analysis was performed in large-scale through the creation of double-mutations and subsequent analysis of colony size (fitness) as previously described in (Tong *et al.*, 2004; Samanfar *et al.*, 2017). In summary, both gene candidates *UGA4* and *YBR062C* were knocked out in MAT α (BY7092) and crossed with two arrays of haploid MAT α knock-out strains (Samanfar *et al.*, 2017). The first array contained 384 deletion strains for genes that are directly or indirectly involved in the process of translation (translation gene deletion array). The second contained 384 random genes that were selected from the YKO collection (Tong *et al.*, 2001; Samanfar *et al.*, 2017), which was used as a control. Selectable markers designated in each background mating type, allowed for multiple selection steps. Meiotic progeny harboring both mutations were selected. The created arrays could then be used to score double mutants for their altered fitness under certain conditions (Tong *et al.*, 2001).

Colony size of both single mutant arrays (reference and control) and double mutant arrays were measured for their colony fitness (Memarian *et al.*, 2007; Wagih *et al.*, 2013). After three repeats, the interactions with 20% alteration or more in at least two repeats were considered positive hits. Conditional SGA analysis was carried on under sub-inhibitory concentrations of chemicals (0.7 mM for As(III) and 60 ng/ml for cycloheximide). PSA analysis was performed under a high sub-inhibitory targeted condition (As(III) (1.2 mM) and cycloheximide (100 ng/ml)) as previously described in

(Tong and Boone, 2007; Samanfar *et al.*, 2017), by overexpressing either *UGA4* or *YBR062C* in the above described translation gene deletion array. Each experiment was repeated three times and the interactions with 25% alteration or more in at least two screens were scored as positive.

3.4 Results and discussion

3.4.1 Deletion of *UGA4* or *YBR062C* makes yeast cells more sensitive to heavy metals and metalloids

The detailed molecular mechanisms that govern cellular responses to heavy metal toxicity and carry out cellular detoxification in living organisms including yeast, have been vigorously investigated over the past decades (e.g. Tamás and Martinoia, 2006; Gadd, 2007; Vadkertiová and Sláviková, 2006; Hosiner *et al.*, 2014). Although much has been learned, additional studies are required to uncover the role of novel genes that may function within these complex networks.

As a result of a large-scale investigation of non-essential gene mutants under heavy metal stress in yeast, we identified potential candidates that may be involved in cellular detoxification of these toxic elements. Two mutant strains for *UGA4* and *YBR062C* illustrate different levels of sensitivity (Fig 3-1A) to three heavy metals (Cd, Ni, and As). In the presence of Cd (0.1 mM), Ni (8 mM), and As(III) (1 mM) in solid media, the number of normalized viable colonies are significantly reduced for both mutant strains (Fig 3-1A), indicating the sensitivity of these mutant strains under the stress caused by heavy metal and metalloid conditions. A complimentary experiment, featuring the reintroduction of all two genes back to their corresponding mutant strains,

showed a complete cellular rescue to heavy metal sensitivity (Fig 3-1B). This cellular recovery, suggests that the observed sensitivity is not the result of secondary mutations within the genome. The sensitivity of these mutant strains to Cd, Ni and As, potentially links their functions to heavy metal toxicity, a novel function previously unreported. These results suggest that *UGA4* or *YBR062C* may either have a direct role in cellular detoxification against heavy metals and metalloids, or that they may have an indirect role by influencing the expression of other genes or other secondary functions involved directly in cellular detoxification.

Similar phenotypic sensitivity to Cd, Ni and As has been previously reported for deletion strain of *URE2* (Todorova *et al.*, 2010; Rai *et al.*, 2003). Given that the Ure2p is a key enzyme involved in yeast heavy metal detoxification (Rai *et al.*, 2003; Urakov *et al.*, 2001), we constructed double gene deletion mutants of *URE2*, and *UGA4* and *YBR062C* respectively to investigate possible interactions involving these genes in metal detoxification. We exposed the double mutant strains to Cd (0.1 mM), Ni (8 mM), and As(III) (1 mM) for further analysis (Fig 3-1C). The sensitivity profile of the double gene deletion strains to the tested metals showed no increase in sensitivity with respect to the phenotypic sensitivity detected for *URE2* single deletion strain (Fig 3-1C). This supports the main effect of *URE2* on heavy metal sensitivity rather than *UGA4* or *YBR062C*. To test for phenotypic compensation of *URE2* function, we overexpressed *UGA4* and *YBR062C* on plasmid vectors in *ure2Δ* background strain (Fig 3-1D). The phenotypic analysis of these strains showed the same levels of sensitivity to Cd, Ni and As(III) with no additional resistance with respect to the background strain *ure2Δ*. These findings suggest that the role of *UGA4* and *YBR062C* in rescuing the cells from the stress caused

by these heavy elements maybe indirect.

According to our data, *UGA4* and *YBR062C* may exert their effect on sensitivity via a parallel pathway to that of *URE2*. This hypothesis of *UGA4* and/or *YBR062C* functioning through a secondary pathway would be supported if an additional increase in sensitivity to Cd, Ni and As(III) occurred when combined with *URE2* deletion, creating a double deletion strains (Márquez *et al.*, 2018). Such a phenotype, however, was not observed in our double deletion sensitivity results (Fig 3-1C). Furthermore, plasmid-based overexpression of *URE2* in *uga4Δ* and *ybr062cΔ* background strains reversed the sensitivity phenotype to Cd, Ni and As(III) observed in the corresponding gene deletions (Fig 3-1E). This result effectively proposes a *URE2*-dependent role of these two genes in heavy metal detoxification process, suggesting that when *URE2* is independently upregulated, the need for *UGA4* and/or *YBR062C* appears to be alleviated. These findings agree with a central role of *URE2* as the critical player in heavy metal and metalloid detoxification, thus suggesting that *URE2* functions downstream of *UGA4* and *YBR062C*.

Figure 3-1: Normalized CFU counts for different yeast strains after exposure to Cd (0.1 mM), Ni (8 mM), and As(III) (1 mM). CFU counts after 3 days exposure to the experimental condition are normalized to control condition counts. (A) Sensitivity of *uga4Δ*, *ybr062cΔ* and *ure2Δ* compare to WT phenotype. (B) Rescued sensitivity of all deletion strains by reintroduction of their overexpression plasmids. (C) Sensitivity analysis for double gene deletions for *UGA4* or *YBR062C* in absence of *URE2* compared to single gene deletion of *URE2*. (D) Sensitivity analysis for overexpression of *UGA4* or *YBR062C* in absence of *URE2*. (E) Sensitivity of *uga4Δ*, *ybr062cΔ* after introduction of pURE2 (carrying *URE2* gene). Error bars represent standard deviation of at least three independent experiments. * Indicates statistically significant differences between WT cells and mutant cells treated with heavy metals (p < 0.005). * Indicates statistically significant differences (t-test) between WT cells and mutant cells treated with heavy metals (p < 0.005). Colour code is the same as in (A) for all panels.

Although both *UGA4* and *YBR062C* have no previously-reported role in metal detoxification, our sensitivity profiles suggest that the influence of *UGA4* and *YBR062C* on heavy metal sensitivity may be connected to expression of *URE2*. The possible mechanism of these two genes in detoxification pathways may be related to the regulation

of either transcription or translation of *URE2*. Further investigation carried out to confirm the details of regulatory mechanism.

3.4.2 The mRNA content of *URE2* remains unaffected by *UGA4* and *YBR062C*

We evaluated possible changes in expression of *URE2* at the mRNA level in absence and presence of *UGA4* or *YBR062C* using RT-qPCR. Our results showed no change in *URE2* mRNA levels when either *UGA4* or *YBR062C* is absent in comparison to WT cells (Fig 3-2). In parallel, since Cd imposed the dominant toxicity with lowest concentration, we chose this toxic element to continue our investigation by evaluating the *URE2* mRNA content also under Cd (0.06 mM) exposure. The fact that deletion of either of these two genes appeared to have no significant influence on the mRNA content of *URE2*, deems their activity independent of *URE2* expression at the mRNA levels.

Figure 3-2: Relative *URE2* mRNA levels quantified by normalization of the *PGK1* mRNA content of mutant strains to those of wild type. *PGK1* mRNA content remained constant and was used to normalize the mRNA levels of *URE2* mRNA in the absence of *ITT1* and *RPS1A* to WT. Deletion of *UGA4* or *YBR062C* had no effect on the normalized *URE2* mRNA content under normal conditions and under exposure to Cd (0.06 mM). Error bars represent standard deviations of at least three trials. The housekeeping gene *PGK1* was used as an internal control.

3.4.3 Relative protein levels of Ure2p are reduced in the absence of *UGA4* or *YBR062C*

The cellular levels of Ure2p in the absence and presence of *UGA4* or *YBR062C* was investigated via western blot analysis. In order to measure the level of Ure2p, a strain of yeast was used which encodes for a GFP fusion protein with a *URE2* ORF controlled under a native promoter. Our western blot analysis showed a reduction in the protein levels of native Ure2-GFP in the absence of *UGA4* or *YBR062C* compare to WT strain expressing both genes (Fig 3-3). Ure2p levels were reduced by approximately 30% and 20%, in *uga4Δ* and *ybr062cΔ* strains, respectively, in comparison to the Ure2p in WT strain (Fig 3-3A). The reduction in protein production of *URE2* suggests that *UGA4* and *YBR062C* may play a role in regulation of Ure2p expression at the translational level. Upon the addition of Cd (0.06 mM) to the growth medium, Ure2p levels were reduced by approximately 80% and 70% in the absence of *UGA4* and *YBR062C* respectively, in comparison to WT strain carrying both genes (Fig 3-3B). Further analysis showed that the absence of *UGA4* or *YBR062C* did not change the translation levels of our housekeeping control, *PGK1*. The observed changes in the translation of *URE2* mRNA in the absence of either *UGA4* or *YBR062C*, connects the activities of both genes to the native levels of Ure2p. Additionally, a substantial reduction for the Ure2p levels in the presence of Cd (0.06 mM) was observed, which suggests that *UGA4* and *YBR062C* may have a significantly higher influence in the regulation of Ure2p expression under oxidative stress conditions induced by heavy metals such as Cd.

Figure 3-3: Western blotting followed by densitometry analysis used to measure native Ure2-GFP levels in different yeast strains. Pgk1p is used as an internal control and values are normalized to those for Pgk1p and are compared to values obtained for WT strain. (A) Cells are grown under standard laboratory conditions. (B) Cells are challenged with Cd (0.06 mM). Each experiment was repeated at least three times. Error bars represent standard deviations. * Indicates statistically significant differences (t-test) between WT cells and mutant cells untreated and treated with As(III) ($p < 0.005$).

3.4.4 Analysis of *URE2* polyribosome-bound mRNA

With the provided evidence suggesting a role for *UGA4* and *YBR062C* in mediating *URE2* expression at the protein level, we performed polyribosome profile analysis to gain insight into the effect of *UGA4* or *YBR062C* on general translation and translation of Ure2p. In this manner, translated mRNAs are separated via ultracentrifugation on a sucrose gradient according to the number of ribosomes attached to them at a time; untranslated mRNAs are at the top and polysome-associated mRNAs are at the bottom. Sample then fractionated and plotted based on their UV absorbance at 254 nm. The profile alignment between gene deletion and WT strains illustrated no significant changes in the profile of free ribosomal subunits (both 40S and 60S) nor in the overall polysome content (Fig 3-4A). This data suggests that *UGA4* and *YBR062C* may

not have an influence on the overall cellular translation profile.

Polysome profiling can also be used for targeted analyses on specific mRNAs. Collected fractions were then processed for total RNA extraction and analysis of the *URE2* polysome-bound mRNAs. In this process, polysomes fractions are isolated and the total RNA is purified for quantification. Next, RT-qPCR is used to evaluate the content of a target mRNA according to its corresponding fraction in the polysome profile. Those mRNAs that are translated more efficiently are generally found in association with a greater number of ribosomes, hence ending up in heavier polysome fractions after separation by intense centrifugation. Those mRNAs which are translated to a lesser degree will therefore be found in lighter fractions according to their sedimentation rate (Shaikho *et al.*, 2016). In this way, the distribution of mRNAs within polysome fractions can be used to estimate the translation efficiency of a targeted mRNA. We used this approach to analyze the polysome-bound mRNA profile of *URE2* in the absence and presence of *UGA4* or *YBR062C*.

Subsequent analysis was carried out using RT-qPCR to quantify the *URE2* mRNA content in each fraction, which was then normalized to an internal control (housekeeping) mRNA (*PGK1*) and plotted in Figure 3-4B. This result, in contrast, illustrated a definitive shift for *URE2* mRNA accumulation towards lighter polysome fractions in the absence of *UGA4* and *YBR062C* (Fig 3-4B), suggesting that *URE2* mRNA is translated less efficiently under those conditions. These data provided evidence that *UGA4* and *YBR062C* directly influence the translation of *URE2* mRNA.

Figure 3-4: Polysome profiles and *URE2* polysome-bound mRNA analysis of *uga4Δ*, *ybr062cΔ* and WT strains (40S, 60S and 80S denote the corresponding free ribosomal subunits and monoribosomes, respectively). A) Alignment of WT polysome profile with *uga4Δ* and *ybr062cΔ* strains. B) The amount of *URE2* mRNA in each fraction was determined by RT-qPCR and the percentage of total *URE2* mRNA on the gradient is plotted for each fraction. The profiles of *PGK1* mRNA were used as an internal control against which to normalize *URE2* mRNA. Each experiment was repeated at least three times. Error bars represent standard deviations.

3.4.5 Ethanol exacerbates Cd sensitivity in *UGA4* and *YBR062C* knockout strains

Since Ure2p is an essential protein for cell survival under physiological conditions such as stress caused by heavy metals, *URE2* mRNA undergoes a cap-independent form of translation in addition to canonical cap-dependent translation, denoting an intricate mechanism of control for *URE2* expression (Komar *et al.*, 2003). Cap-dependent translation is initiated via the attachment of the 40S ribosomal subunit to the 7-methylguanosine cap on the mRNA 5' UTR, and is mediated through the scanning of the mRNA in order to position the ribosome at the most suitable AUG initiation codon. In contrast, in internal initiation of translation, the interaction between 40S ribosomal subunit and mRNA is directly mediated via mRNA structures known as IRESs, which operate independently of the 7-methylguanosine cap (Holcik and Sonenberg, 2005). This method of translation initiation is mainly utilized by viruses but can also be found in

some eukaryotic mRNAs (Pelletier and Sonenberg, 1988; Liwak *et al.*, 2012). Although the translation initiation in eukaryotic cells primarily takes place via cap-dependent translation, whenever general translation may be compromised (e.g. under physiological stress conditions), translation of some mRNAs is often mediated via an IRES element (Stoneley and Willis, 2004; Holcik and Sonenberg, 2005; Komar and Hatzoglou, 2011).

Previous studies suggested that under stress conditions, canonical cap-dependent form of translation initiation maybe inhibited where IRES-dependent translation may not be affected (Johnson *et al.*, 2017). Under these conditions, essential cellular mRNAs, responsible for proteins that are involved in the stress response, especially apoptosis and hypoxia, naturally contain IRES elements to overcome such stress conditions (Silva *et al.*, 2013; Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018). Previous studies suggest that exposure to heavy metals applies such stress conditions on yeast cells (e.g. Spriggs *et al.*, 2008; Todorova *et al.*, 2010). By also considering the fact that *URE2* forms IRES structure on its mRNA, we examined whether *UGA4* and *YBR062C* somehow influence IRES-mediated translation of *URE2* mRNA. Cellular stress in the form of exposure to ethanol has been used as a positive control for experiments examining the IRES-mediated translation of *URE2* via changes in the function of eukaryotic Initiation Factor 2A (eIF2A) as a regulator of IRES-mediated translation in yeast cells (Reineke *et al.*, 2011). IRES-mediated translation of *URE2* is the preferred mechanism during the time eIF2A remains inactive, a condition facilitated by exposure of cells to 6% ethanol (Reineke *et al.*, 2011).

As the critical role of *URE2* in heavy metal detoxification was previously noted ((Todorova *et al.*, 2010; Komar and Hatzoglou, 2011), exposure to 6% ethanol may cause additional distress to the sensitivity of *uga4Δ* and *ybr062cΔ* to Cd treatment, if *UGA4* and *YBR062C* are the regulators of *URE2*-IRES translation. In this case, ethanol can introduce a dual effect on the same overall process in the absence of either *UGA4* or *YBR062C*.

We first exposed *ure2Δ*, *uga4Δ* and *ybr062cΔ* strains to a mild concentration (lower sub-inhibitory) of Cd (0.04 mM) and 6% ethanol separately, treatments which did not produce a sensitive phenotype (Fig 3-5). On the contrary, combination of both Cd as low as 0.04 mM and 6% ethanol together resulted in hypersensitivity, which suggests that ethanol increases the sensitivity of these deletion strains to Cd (0.04 mM) (Fig 3-5). These results may demonstrate a likely connection between the functions of *UGA4* and *YBR062C* and the translation of *URE2* through its internal mRNA structure. However, these outcomes are open to other explanations, for instance, ethanol and Cd may impose alternative effects on the cell viability independent of IRES-mediated translation. In this context double deletion strains for *eIF2A* with *UGA4* or *YBR062C* illustrated similar sensitivity to the presence of either a low concentration of Cd (0.04 mM) alone or with ethanol (6%) (Fig 3-5) linking the reported activity of ethanol to *eIF2A*. Nonetheless, further experiments to directly examine the *URE2*-IRES are required to confirm these elucidations. In this context, we also examined the sensitivity of double mutant strains for *UGA4* and *YBR062C* with *URE2* to the three tested conditions. This did not result in extra sensitivity compared to *ure2Δ* sensitivity (Fig 3-5). These findings yet again link the activities of *UGA4* and *YBR062C* to the expression of *URE2*.

Figure 3-5: Viability of single and double gene deletion strains for *UGA4* and *YBR062C* with *URE2*. Cells were exposed to Cd (0.04 mM), 6% ethanol, a combination of both (6% ethanol + Cd (0.04 mM)). Standard growth medium with no treatment was used as a control. Average CFU counts after 3 days of the experimental conditions are normalized to average CFU counts for WT strain and presented as a percentage of the average CFU counts. Each experiment was repeated at least three times. Error bars are calculated as standard deviations. * Indicates statistically significant differences (t-test) between WT cells and mutant cells treated with As(III) + ethanol ($p < 0.005$).

3.4.5 Deletion of *UGA4* or *YBR062C* reduced IRES-mediated translation of *URE2*

Based on our sensitivity results in the presence of ethanol, which gave us a clue about the probable role of *UGA4* and *YBR062C* on IRES-mediated translation of *URE2*, we further investigated their direct involvement in the IRES-mediated translation of *URE2*. We employed a plasmid-based experiment that prevents canonical 5'cap-initiated translation using a series of strong inhibitory structure (hairpin loops) in the 5' UTR and instead monitors a β -galactosidase reporter under the control of the *URE2*-IRES element (Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018). A construct called p281-4-*URE2* was used to measure the protein expression of the reporter gene through cap-independent translation via the *URE2*-IRES element portion of Ure2p in the presence and absence of

UGA4 and *YBR062C* (Fig 3-6) A control plasmid (p-281) lacking the hairpin structure and thus allowing cap-dependent translation of the reporter was also used as a control (Komar *et al.*, 2003; Reineke *et al.*, 2011).

Figure 3-6: Loss of *UGA4* and *YBR062C* decreases the ability of the *URE2*-IRES element to initiate cap-independent translation. (A) Plasmid-based reporter assay indicates that *uga4* Δ and *ybr062c* Δ strains have no effect on cap-dependent translation but significantly decrease the efficiency of *URE2*-IRES cap-independent translation. Relative β -galactosidase activity was determined by normalization of the activity of the deletion strains to that of the WT strain. Blue bars represent β -galactosidase activity under the translational control of *URE2*-IRES. Red bars represent β -galactosidase activity via normal translational control of the 5' cap. (B) Deletion of *UGA4* or *YBR062C* does not affect the mRNA content of the β -galactosidase reporter. The relative mRNA levels of β -galactosidase are quantified by normalizing the mRNA content of the *URE2* in mutant strains to the wild type strain. *PGK1* mRNA (housekeeping gene) was used as an internal control to normalize the mRNA levels of β -galactosidase mRNA in WT, *uga4* Δ and *ybr062c* Δ . Deletion of *UGA4* or *YBR062C* introduced no change in the normalized β -galactosidase mRNA content. Error bars are calculated as standard deviations from at least three separate experiments. * Indicates statistically significant differences (t-test) between WT cells and mutant cells ($p < 0.005$).

In this experiment, the absence of *UGA4* and *YBR062C* resulted in reduced translation of β -galactosidase mRNA under the translational control of the *URE2*-IRES element. This result provides additional support for a regulatory role of *UGA4* and

YBR062C towards the translation of *URE2* via its IRES element. Quantification of β -galactosidase activity did not show a significant difference for *uga4 Δ* , *ybr062c Δ* and WT strains, which suggests that *UGA4* and *YBR062C* may not play a substantial role in influencing cap-dependent translation of *URE2* mRNA (Fig 3-6). Furthermore, to test whether the reduced levels of β -galactosidase are a consequence of reduced mRNA content, the mRNA of β -galactosidase expression was inspected using RT-qPCR. This experiment did not result in any significant change in β -galactosidase mRNA content in the absence or presence of the investigated genes (Fig 3-6B).

3.4.6 Genetic interaction analysis further links the activity of *UGA4* and *YBR062C* to regulation of translation under Cd and cycloheximide stress conditions

We further investigated the translational role of *UGA4* and *YBR062C* by analyzing their genetic interaction (GI) network under normal and stress conditions such as cycloheximide and Cd. Cycloheximide is an antibiotic that binds to the E-site of the ribosome and affects deacylated tRNA to inhibit general cellular translation (Schneider-Poetsch *et al.*, 2010). Target genes were crossed with an array of 384 genes associated with post-transcriptional regulation of gene expression and a control array with 384 randomly-selected genes as a control. Functionally related genes are usually form epistatic interactions (Rieger *et al.*, 2012; Szendro *et al.*, 2013). The most well studied form of these kind of interactions is known as negative GI, where decreased fitness or a lethal phenotype is observed in a double-mutant beyond what is observed when each gene is deleted individually (Samanfar *et al.*, 2017). These genes are often shown to associate with compensating-parallel pathways and analysis of negative GIs can provide valuable insight into novel gene function (Tong *et al.*, 2004; Samanfar *et al.*, 2017).

UGA4 formed negative GIs under standard growth conditions with only three genes involved in the process of protein biosynthesis (Fig 3-7A) whereas *YBR062C* forms no negative GIs under the same condition (Fig 3-7B). Both genes formed limited number of negative GIs with other pathways such as catabolic process and helicase activity (Figs 3-7A and B). For instance, *UGA4* formed negative GIs with *YOR302W* which was shown to have regulatory role in translation of the *CPA1* mRNA (Delbecq *et al.*, 1994), and *YBR062C* formed negative GIs with two heat shock proteins, *HSP82* and *HSC82*. Both heat shock proteins are paralogs and cytoplasmic chaperone of the *HSP90* family which are essential for high temperature conditions (Finkelstein *et al.*, 1983; Borkovich *et al.*, 1989; Byrne *et al.*, 2005).

Another type of GI is formed under physiological stress conditions known as conditional GIs. These types of interesting GIs provide a more focussed understanding on the function of a given gene under specific conditions (Babu *et al.*, 2011; Samanfar *et al.*, 2017). They characterise the mosaic nature of gene function(s) which can be altered due to different external or internal physiological stimuli. For example, the expression of many known genes that function in DNA damage repair are differentially regulated in response to DNA damaging agents (Tong and Boone, 2007; Omid *et al.*, 2014). In this study, we analyzed the negative GIs formed by *UGA4* and *YBR062C* under stress caused by sublethal concentrations of cycloheximide (60 ng/ml) and Cd (0.08 mM). Under both conditions, both genes formed a novel set of interactions with several genes that primarily play a role in the post-transcriptional regulation of gene expression (Figs 3-7A and B). Our results propose that in the presence of cycloheximide and Cd, both genes seem to fulfil a novel role in regulating the process of protein biosynthesis (p-value *UGA4*

cycloheximide = 4.17×10^{-7} , *UGA4* Cd = 1.21×10^{-9} and p-value *YBR062C* cycloheximide = 5.80×10^{-4} Cd = 6.17×10^{-19}) (Figs 3-7A and B).

Among the genes that formed a novel negative genetic interaction in the presence of Cd with both *UGA4* and *YBR062C*, there are several interesting candidates that function as regulators of translation such as *NPL3* and *HRC1*. Nuclear Protein Localization 3 (*NPL3*) encodes an RNA-binding protein that promotes elongation, regulates termination, and carries poly(A) mRNA from nucleus to cytoplasm (Ott *et al.*, 2006). Npl3p represses translation initiation by binding with eIF4G, it is also required for pre-mRNA splicing and may have role in telomere maintenance and is essential for optimal translation during nutrient deficiency (Windgassen *et al.*, 2004; Rajyaguru and Parker., 2012). High-Copy suppressor of Rpg1 *HCR1* is another example of the negative interactions gained by *UGA4* and *YBR062C* under Cd-stress condition. Hcr1p is a component of translation initiation factor 3 (eIF3) that binds eIF3 subunits Rpg1p, Prt1p and 18S rRNA (Beznosková *et al.*, 2015). Hcr1p holds dual functions in the regulation of translation. It is involved in translation initiation as a sub-stoichiometric component (eIF3j) of eIF3. It directly binds to mRNA substrates and activates mRNA decapping which is required for 20S pre-rRNA processing and at post-transcriptional step for efficient retro-transposition (Valášek *et al.*, 2001). Hcr1p also play a role in translation during stress conditions such as heat shock (Valášek *et al.*, 1999).

To provide a more comprehensive analysis of the interaction network of candidate genes under translational stress, negative GIs induced by eukaryotic translational inhibitor drug; cycloheximide was also studied. In this case cycloheximide can suppress translation

and cause defect in the recruitment of deacylated tRNA to the E-site of the ribosome (Schneider-Poetsch *et al.*, 2010). This can impose more targeted stress on translation in addition to double gene deletions, which in return reveals more genes that are competing with *UGA4* and/or *YBR062C* in rescuing translation during conditions such as cycloheximide exposure (Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018). This analysis complemented the negative GIs detected under Cd-challenge with *UGA4* and *YBR062C* again forming common novel negative GIs with a number of genes that are associated with the regulation of translation following the introduction of a relatively-low concentration of cycloheximide (60 ng/ml) (p-value *UGA4* = 2.38e-4 and p-value *YBR062C* = 5.80e-4). Translation Initiation Factor 4A (*TIF1*) and its paralog *TIF2* are examples of the gained interaction for both *UGA4* and *YBR062C* in response to cycloheximide. Tif proteins are key players in translation initiation that interact with eIF4G and hold a helicase activity (Linder and Slonimski, 1989). These types of shifts in GIs networks (e.g. Samanfar *et al.*, 2017; Omid *et al.*, 2017; Moteshareie *et al.*, 2018) strongly suggest the involvement of both *UGA4* and *YBR062C* in regulating translation in response to stress conditions.

Figure 3-7: GI network for *UGA4* and *YBR062C*. (A) Negative GIs for *UGA4* under standard growth condition and the presence of Cd (0.08 mM) or cycloheximide (60

ng/ml). In this case, deletion of a second gene along with *UGA4* forms an unexpected defective phenotype. (B) Negative GIs for *YBR062C* under standard growth conditions and the presence of Cd (0.08) and/or cycloheximide (60 ng/ml) as in (A). Each experiment was repeated three times and the interactions with more than 25% alteration in at least two screens were scored as positive hit. Network analysis and associated P-values were obtained from GeneMANIA.

3.4.7 Phenotypic suppression analysis further links the activity of *UGA4* and *YBR062C* to regulation of translation under Cd and cycloheximide stress conditions

Phenotypic suppression analysis (PSA) is another technique for understanding functional genetic relationships under specified stress conditions. PSA can infer more direct functional relationships between interacting genes by establishing a more direct form of GI (Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018). It refers to situations where defective growth caused by deletion of a particular gene, in a specific growth condition (for example presence of a drug) is rescued by the overexpression of another (Alamgir *et al.*, 2010; Samanfar *et al.*, 2017). PSA fitness screening was employed to assess if the overexpression of the target genes can rescue (suppress) the sensitive or lethal phenotypes of single mutants on our two sets of 384 mutant arrays under translational stress conditions (Alamgir *et al.*, 2008; Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018) (Figs 3-8A and B).

Our PSA screen detected that the sensitivity of a number of gene deletion strains to Cd (0.13 mM) or cycloheximide (100 ng/ml) was rescued by overexpression of either *UGA4* or *YBR062C* individually (Figs 3-8A and B). Most of the novel interacting partners that are detected under the experimental stress conditions are involved in translation regulation (Figs 3-8A and B). This observation further connects the activity of *UGA4* and *YBR062C* to the regulation of translation under tested stress conditions. Remarkably, our

PSA analysis detected three novel interacting partners, *GIS2*, *ITT1* and *DOM34* that previously have been reported to have IRES trans-acting factor (ITAF) activity Scherrer *et al.*, 2011; Sammons *et al.*, 2011; Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018). The fact that the overexpression of *UGA4* and *YBR062C* can compensate for the absence of these three known ITAFs, provides further evidence that connects them to IRES activity.

Glg Suppressor (*GIS2*) is known to activate several mRNAs that can be translated via their IRES region in yeast (Scherrer *et al.*, 2011; Sammons *et al.*, 2011). In addition to our genetic interaction analysis, a physical interaction between Ybr062cp and Gis2p is also previously reported (Sammons *et al.*, 2011) that further connects the functional activity of these two proteins. Inhibitor of Translation Termination 1 (*ITT1*) is another interacting partner of *YBR062C* from our PSA analysis that is previously shown to play a role in IRES-mediated translation of *URE2* (Moteshareie *et al.*, 2018) which suggests a close relationship between *YBR062C* and *ITT1* in controlling IRES-mediated translation of *URE2*. Duplication Of Multilocus region 34 (*DOM34*) was also a positive hit that encodes for a protein that functions in dissociation of mRNA attached inactive ribosomal subunits to aid in translation restart as an mRNA quality control (Davis et al.1998; Passos *et al.*, 2009), and is also reported to have ITAF activity in yeast (Samanfar *et al.*, 2017).

Figure 3-8: GI network for *UGA4* and *YBR062C*. (A) PSA analysis utilizing a plasmid harboring *UGA4*. In this way, overexpression for *UGA4* rescued the sensitivity of gene

deletions to Cd (0.13 mM) or cyclohexamide (100 ng/ml). (B) PSA analysis utilizing a plasmid expressing *YBR062C* as in (A). Each experiment was repeated three times and the interactions with more than 25% alteration in at least two screens were scored as positive hit. Network analysis and associated P-values were obtained from GeneMANIA.

Combined, the results of PSA analysis suggest a role for both *UGA4* and *YBR062C* in regulation of IRES-mediated translation. Moreover, this data, in agreement with our previous study (Moteshareie *et al.*, 2018), proposes a systematic compensation between certain ITAFs which can be the subject of further investigations. As it was also previously proposed (Moteshareie *et al.*, 2018), PSA analysis also leads to the conclusion, that other novel genetically interacting partners of *UGA4* and *YBR062C*, may be involved in internal initiation of translation. Further studies are required to address these hypotheses.

4 Chapter 4: Large-scale investigation of multiple IRES elements in yeast, *Saccharomyces cerevisiae*

4.1 Abstract

Internal ribosome entry sites (IRESs) are structural elements formed on certain viral and eukaryotic RNAs that are capable of initiating protein synthesis by direct recruitment of 40S ribosomal subunit, free of the 7-methylguanosine cap at the 5' end of the mRNA. This direct recruitment requires combinations of different factors, including canonical factors and IRES specific factors to cross-communicate with the components of translation machinery to mediate the internal initiation of translation. Although a wealth of information exists on viral IRESs, eukaryotic IRESs remain elusive given the little structural congruence observed between multiple cellular IRES elements. As a powerful tool systems biology has provided excellent opportunity to study these elements according to their regulatory networks rather than morphological features. In this chapter of this project, we sought out to identify novel genes encoding regulatory factors known as IRES *trans*-acting factors (ITAFs). Utilizing systems biology techniques for functional genomics investigation into ITAFs roles, we performed a high-throughput plasmid-based screening of a library of non-essential gene deletion strains, using baker's yeast (*Saccharomyces cerevisiae*) as our model organism. To this end, we constructed 12 plasmids each containing a single eukaryotic IRES element fused to a β -galactosidase reporter. Five of these constructs showed constitutive expression and were used to challenge a collection of more than 4300 non-essential gene knockout strains of *S. cerevisiae*. We identified 142 genes out of which five were observed to be involved in regulating at least three or more IRES elements and were hence selected for further experimental analysis. Quantitative β -galactosidase assays also showed lower expression levels for all corresponding IRES elements in the absence of these genes. RT-qPCR was used to confirm that their regulatory involvement was not transcriptional in nature. This was then followed by western blot analysis to examine the endogenous protein levels which also displayed lower translation levels for all corresponding IRES elements in the absence of these genes. With these newly identified ITAFs, we hope to incite future research aiming to elucidate distinct IRES mechanisms shared by different cellular IRES elements.

4.2 Introduction

Conversion of mRNA sequences to proteins is one of the most fundamental cellular processes and a key element in the central dogma of molecular biology, making it essential to maintain the homeostasis of a cell or an organism. Also known as protein synthesis, this process involves interactions between different components of translation machinery; large ribonucleoproteins known as ribosomes, and a wide array of accessory proteins and mRNAs (Schuller and Green, 2018). Ribosomal machinery is first assembled by recruiting a transfer RNA (tRNA) molecule, specialized for initiation (Met-tRNA_i) carrying initiator methionine amino acid in a step known as translation initiation (Kapp and Lorsch, 2004; Schuller and Green, 2018). Translation initiation enters the elongation step when the ribosome is correctly aligned the Met-tRNA_i with the AUG start codon (Jackson *et. al.*, 2010). This involves the dissociation of most of initiation factors, and the subsequent recruitment of 60S ribosomal subunit and formation of the 80S initiation complex (80S-IC) (Hinnebusch, 2014). During the elongation step, the ribosome moves down the messenger RNA (mRNA) in a processive manner from 5' to 3' direction, decodes it with the recruitment of aminoacyl tRNA molecules, and catalyzes the formation of peptide bonds between the different amino acids brought to the translation machinery (Kapp and Lorsch, 2004). The final step of translation process is called termination which occurs upon recognition of a stop codon (UAG, UAA, or UGA). At this time, proteins called release factors enter the A-site of the ribosome and by forming a heterodimer, they can stop the translation and release the fully-grown polypeptide chain. This process then disassembles the ribosome and its associated factors for either recycling or another round of translation (Kapp and Lorsch, 2004). Due to its dynamic process, the

major regulation of protein synthesis is exerted at initiation level, therefore the molecular basis of this process is currently under extensive study. Initiation of translation constitutes one of the most fundamental and ample target processes for the regulation of protein synthesis and as the first step in the overall process of translation, it offers a high regulatory potential and targeting it allows for a quick and reversible expression control at the cellular level (Jackson *et. al.*, 2010).

For several decades, the canonical 7-methylguanosine cap-dependent translation was the only known way of translation initiation. In contrast to cap-dependent translation, cap-independent translation via IRES does not require the canonical 7-methylguanosine cap nor associated factors. IRES-mediated translation was initially discovered in picornaviruses by Pelletier and Sonenberg in 1988, this form translation initiation can take place in the mRNAs of viruses like hepatitis C (HCV). Soon after the discovery of viral IRESs, they were also discovered in eukaryotic cells during stress conditions such as hypoxia, apoptosis, or starvation (King *et. al.*, 2010; Johnson *et al.*, 2017). Many viruses such as the family of *Picornaviridae* viruses including picornaviruses can bypass the essential need for cap structure at the 5' end of their RNAs which having IRES element gives them an ability to freely replicate their uncapped RNA genomes within a cell (King *et. al.*, 2010). During infection by IRES containing viruses, the canonical form of translation is inhibited, largely as a result of the cleavage of eIF4G by viral proteases (Sweeney *et. al.*, 2013). The ability of viral IRESs to initiate the translation of their own mRNA was supported by the observation that all the components of the cap-binding complex, eIF4F, were not necessary for successful translation of the EMCV and polio viral genomes (Pelletier and Sonenberg, 1988; King *et. al.*, 2010).

The structures formed by IRESs, allow the 40S ribosomal subunit to directly bind to the IRES element of an mRNA, thereby more directly and efficiently bypassing unfavourable AUGs (Komar *et. al.*, 2012). Soon after discovery of viral IRESs, first IRES structure in eukaryotic cells was observed as the constant expression of binding immunoglobulin protein (BiP) during a picornavirus infection where cap-dependent initiation of translation was shut down (Macejak and Sarnow, 1991). This notion first supports that IRES-mediated translation appears to take place during stress conditions where the cap-dependent mechanism is impaired or inhibited which is reasonable to postulate that gene products under IRES control primarily may perform cellular functions related to growth regulation and cell viability (King *et. al.*, 2010).

During recent years, number of cellular IRES elements have been rapidly multiplied, and it has been estimated that during stress conditions where canonical initiation of translation is inhibited, 10% of cellular mRNAs are under some sort of structural-based internal recruitment of ribosomal subunits that can bypass this inhibition (King *et. al.*, 2010). IRES-mediated way of translation initiation is regulated by certain canonical initiation factors, as well as additional proteins known as IRES trans-acting factors or ITAFs (King *et. al.*, 2010; Yamamoto *et. al.*, 2017). Majority of ITAFs perform functions in pathways unrelated to initiation process of translation. For example, maximum efficiency in translation of the EMCV viral IRES element requires pyrimidine tract binding protein (PTB) which serves as an ITAF (Komar *et. al.*, 2012). A common mechanism of action for ITAFs has been suggested to stabilize IRES elements through RNA chaperone activity (King *et. al.*, 2010; Komar *et. al.*, 2012). This also supports the possibility of internal structural modifications which may facilitate a better interaction

site on the IRES structure for the ribosome and/or additional canonical factors to more easily be recruited (King *et al.*, 2010; Komar *et al.*, 2012). In contrast, ITAFs can also downregulate the process for other loci, meaning, ITAFs can positively or negatively alter the efficiency of IRES-mediated translation (King *et al.*, 2010; Johnson *et al.*, 2017). Interestingly, there exist several cellular ITAFs that can be translated through IRES-mediated translation, with other ITAFs playing roles in regulation of their translation (King *et al.*, 2010).

Viral IRES structures, based on their structure and requirement factors are categorized into four simple known types. Viral IRES structures are well studied and characterized, each with its own unique sequences, structures, and sets of ITAF requirements. Whereas cellular IRESs, due to their complexity, structural diversity and variation of required factors, remained uncharacterized, even after more than two decades of vigorous investigations (Lozano and Martinez-Salas, 2015; Jackson *et al.*, 2010). IRESs can also be found in intergenic regions, a phenomenon which is also observed in eukaryotes (Johnson *et al.*, 2017). Other cellular IRES elements can be found within protein-coding sequences (Ray *et al.*, 2006). For example, the cap-dependent translation of *URE2* gene is 94 amino acids larger than cap-independent translated form that possesses prionogenic properties and is often referred to as the Ure3p form (Reineke *et al.*, 2011). Another example can be found in the human gene coding for p53 tumour suppressor protein which has been shown to possess two IRES elements, one of which is located in the coding region (Ray *et al.*, 2006).

Since under certain stress conditions, canonical initiation of translation is

compromised, survivor of a cell depends on IRES-mediated translation. Understanding how eukaryotic cells are able to overcome different pathophysiological stress states, we need to investigate and better understand how exactly cellular internal initiation works and what are the associated factors involved in this process (King *et. al.*, 2010; Komar and Hatzoglou, 2011; Johnson *et. al.*, 2017). Cancer cells are clear example of how cells can survive and replicate in stress conditions such as hypoxia and nutrient deficiency (Eales *et. al.*, 2016). Tumor cells are capable of surviving and replicating under conditions that would normally lead to several complications in healthy cells (Eales *et. al.*, 2016; Komar and Hatzoglou, 2015). Evidently, certain genes for survival of cancerous cells may be expressed through IRES-mediated translation (Komar and Hatzoglou, 2015). Indeed, studying cellular IRESs and how to regulate them, first and foremost requires investigation of cellular ITAFs and closer look into their roles in this process. A comprehensive investigation into the ITAF networks and their associated IRESs, could enable the design or screening of small compounds that may be able to inhibit the function of certain ITAFs to regulate specific IRES-mediated translation of certain IRESs which can have pharmaceutical applications (Komar and Hatzoglou, 2015). Furthermore, a systematic investigation into the role of novel ITAFs that generally control several IRES elements or exploring very essential novel ITAFs that can affect the general process of IRES-mediated translation, can also aid in controlling the general or a certain group of IRESs. One study claimed that the blockage of IRES-mediated translation of human amyloid precursor protein (APP) might be a new high-level specificity and inoffensive treatment for Alzheimer's disease (AD) (Qy Liu, 2015). It is noteworthy that knowing the specific mechanisms and ITAFs that control IRES-mediated

translation of APP is significantly important in this case.

Unfortunately, to date, there is a deficit of studies revealing any degree of structural similarity between different cellular IRES elements in the literature (Yamamoto *et. al.*, 2017). Despite the abundance of literature concerning the structure of viral IRESs, the detailed mechanisms by which they function have not yet been properly investigated nor understood (Komar and Hatzoglou, 2011; Johnson *et al.*, 2017). There is hence an opportunity for new researchers in the field of IRES-mediated translation, to investigate and ultimately start classifying eukaryotic IRESs using powerful systems biology and computational tools that help overcome the challenges that are suppressing this fascinating field of study thus far.

As an ideal model organism, baker's yeast (*Saccharomyces cerevisiae*) offers unique genetic tools that facilitate such approaches to systematically investigate eukaryotic IRESs in addition to their specific and/or generally associated ITAFs (Komar *et. al.*, 2012). Library of different arrays including different deletion arrays, overexpression arrays and protein tag fusion arrays in yeast facilitates systematic experimental tools that can be used to investigate functional genomics (Reid *et. al.*, 2016). We used a plasmid-based screening that uses the underlying principle of large-scale transformation to transform a collection of yeast knockout (YKO) mutants (Tong *et. al.* 2001). We used this large-scale technique to identify novel ITAFs involved in the non-canonical translation initiation of multiple IRES elements. The main purpose of this project is to identify genes that code for proteins with a previously undiscovered role as an ITAF that can specifically or generally control the selected IRES elements. In this

experiment, the YKO collection library can be challenged by a plasmid containing a region known to contain IRES fused to a reporter gene, such as *β-galactosidase* reporter gene (Komar *et al.*, 2012). A previously known plasmid called p281-4 was used in this project that features a series of four highly stable hairpin loops at the 5' end which serve to inhibit cap-dependent initiation mechanism of translation and instead allow only for internal initiation to take place which is positioned right after the four hairpin loops (Komar *et al.*, 2003). A yeast deletion strain showing a reduction or induction in the reporter activity indicates its role, respectively, in IRES-mediated translation of the tested IRES element. By utilizing this procedure, this project had the primary aim of identifying novel genes involved in downregulating the IRES-mediated translation of 13 known IRES elements, 11 in yeast which included all previously identified known yeast IRESs that have not been used in earlier studies for large-scale investigation to identify potential regulatory candidates (e.g. *HSP82* and *HSC82* were previously investigated by Bahram *et al.*, in 2015). To also gain more insight about human IRESs, we additionally selected two human IRESs to be used in our investigations.

The human p53 mRNA contains two distinct IRES elements, one in 5' UTR and the other in its coding region (Ray *et al.*, 2006). The p53 internal structures lead to the full length and N-terminally truncated p53 isoforms respectively (Ray *et al.*, 2006). All yeast IRES elements were either found in the online database IRESite or well-documented in the literature. For instance, Yeast AP-1 (*YAPI*), encodes for a transcription factor which is involved in the oxidative stress response (Moye-Rowley *et al.*, 1989; Tkach *et al.*, 2012), and SuPpressor of Ty insertions (*SPTI5*), encodes for the yeast TATA-binding protein (Kobayashi *et al.*, 2003; Gruhlkea *et al.*, 2017). Ensuing

identification of both specific and general ITAFs, we followed our study by validating the involvement of five ITAFs identified in common between at least four or more IRESs by conducting gene expression experiments, utilizing yeast, *S. cerevisiae*. To this end, the gene expression analysis facilitated us to establish a better connection between the targeted IRESs and the associated ITAFs.

4.3 Aims and objectives

Our first objective was to sub-clone new IRES forming regions into a well-studied plasmid known as p218-4-*URE2* (Komar *et al.*, 2003; Moteshareie *et al.*, 2018), replacing *URE2* with twelve different IRES elements. After construction of new plasmids with 12 new IRES regions, we would like to use these plasmids in a large-scale screening to identify novel genes exhibiting potential ITAF activity towards each of our targeted IRES elements. We would then like to follow up our investigations with candidate genes sharing four or more IRES elements to be considered as general ITAFs. This chapter will be finalized by further investigations into the role of these general ITAFs, using gene expression analysis via quantitative experiments. Our findings can contribute to provide a platform for future experiments to determine whether any common mechanism exists for the regulation of cellular IRES-mediated translation and ultimately, proposing a possible classification based on their regulatory networks rather than structural features.

4.4 Materials and methods

4.4.1 Strains and plasmids

Yeast, *S. cerevisiae*, mating type (a) *MATa* strain Y4741 (*MATa orf1::KanMAX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and mating type (α) *MATα* strain, BY7092 (*MATα can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu21Δ0 ura3Δ0 met15Δ0*) (Tong *et al.*, 2001) were utilized for this project. The yeast *MATa* knockout (YKO) collection (Tong *et al.*, 2001) was used for large-scale screening. A PCR-based technique was used to create 6HIS fusion for western blot analysis. pAG25 plasmid containing the Nourseothricin Sulfate (clonNAT) resistance gene was used as a DNA template in PCR to generate 6HIS fusion strains. *Escherichia coli* strain *DH5α* was used to replicate different plasmids (Taylor *et al.*, 1993). Two plasmids were used carrying the β -galactosidase open reading frame for quantification of *URE2*-IRES and cap-dependent translation activities. p281-4-*URE2* contained a *URE2*-IRES region which was fused with *LacZ* (β -galactosidase) gene from *E. coli*, and p281 contained only the β -galactosidase gene as a control for cap-dependent translation (Komar *et al.*, 2003). All shuttle plasmids carried an ampicillin resistance gene which was used as selectable marker in *E. coli* and the *URA* requiring 3 (*URA3*) marker gene which was used for selection in yeast.

4.4.2 Media and reagents

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. For all media used during the high-throughput screening (large-scale transformation), please refer to Tong and Boone, 2006. Yeast cells were grown at 30°C unless otherwise stated. *E. coli* cells were grown at 37°C. Yeast plasmid extraction was performed by using yeast plasmid mini kit (Omega Bio-tek®) and *E. coli* plasmid extraction was carried out by using GeneJET plasmid miniprep kit (Thermofisher®) according to the manufacturer's instructions.

4.4.3 Cloning IRES elements and plasmid construction

The online resource Saccharomyces Genome Database (SGD) used to obtain the yeast gene sequences to design oligonucleotide primers for the subcloning process of the ten *Saccharomyces cerevisiae* IRES forming regions into the p281-4-*URE2* plasmid, replacing with *URE2*. The NCBI BLAST® was used to design primers for the two p53 IRES regions. In this project, partial region covering the entire IRES region was used instead of the entire gene, as they are of excessive length for efficient subcloning. Restriction enzyme target sequences for *SacII* and *EcoRI* were introduced at the 5' end of the forward and reverse primers, respectively. Full primer sequences are available in Appendix 4.A. DNA sequences of the IRES elements of interest were PCR-amplified from wild type *S. cerevisiae* genomic DNA (gDNA) to confirm the proper functioning and specificity of the primers. Total cDNA samples derived from tunicamycin-treated HeLa cells were used to amplify the two IRES elements of the human p53 gene, p53 and p53ΔN (Ray *et. al.*, 2006). After confirmation, IRES regions of interest were amplified through PCR and amplicons were then purified using GeneJET PCR Purification Kit

(Thermo Fisher Scientific) according to the manufacturer's instructions. p281-4-*URE2* plasmid was extracted from previously transformed DH5 α *E. coli* cells. All *E. coli* plasmid extractions were performed using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). P281-4-*URE2* plasmid was digested with *Sac*II and *Eco*RI restriction enzymes for 3 hours at 37°C, and 20 minutes at 65°C for inactivation of the enzymes. All restriction endonuclease and ligase enzymes used in this study, and their pertinent buffers were obtained from New England Biolabs Ltd. (NEB). Due to the nature of their design, IRES region purified-amplicons were digested with *Sac*II and *Eco*RI for 3 hours at 37°C and 20 minutes at 65°C for inactivation. Agarose gel was used to visualize and confirm each restriction digest. These bands were subsequently purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific). IRES-DNA regions were subcloned into p281-4 plasmid by ligation using T4 DNA ligase enzyme overnight at 16°C and 10 minutes at 65°C for inactivation. All p281-4-IRES recombinant plasmids were transformed into competent DH5 α *E. coli* via a heat shock transformation method. Transformed colonies were confirmed using colony PCR with the same primers designed for IRES region amplification. The corresponding bands to the IRES regions were confirmed using agarose gel electrophoresis. Finally, in order to verify the sequences and proper plasmid constructions, the subcloned plasmids were sent for DNA Sanger sequencing to Bio Basic, Inc. (Markham, ON, Canada). Amplifications took place in the forward and reverse directions using the same sequence-specific primers as for IRES region amplification. Full sequencing results are available in Appendix 4.B.

4.4.4 Quantitative β -galactosidase assay

ortho-Nitrophenyl- β -galactoside (ONPG)-based β -galactosidase analysis was used to quantify involvement of the identified genes in the IRES-mediated translation of *URE2* via quantification of β -galactosidase activity produced by plasmids containing targeted IRES regions fused to a β -galactosidase reporter (p281-4-IRES) (Komar *et al.*, 2003). This plasmid contains a DNA sequence that forms four strong hairpin loops prior to the IRES region at its mRNA level. These four hairpin loops inhibit cap-dependent translation of β -galactosidase mRNA. A background plasmid (p281) carrying only a β -galactosidase reporter was used as a control for cap-dependent translation (Komar *et al.*, 2003). Both plasmids contained a GAL1/10 promoter and YPG was used as a medium to activate the desired gene (Samanfar *et al.*, 2014). Each experiment was repeated at least three times.

4.4.5 Large-scale transformation and confirmation of the query plasmids

Plasmid transformations for MAT α were carried out by using a chemical-transformation strategy (LiOAc method) and confirmed via colony PCR (Alamgir *et al.*, 2010; Samanfar *et al.*, 2017). Confirmatory β -galactosidase activity assays were carried out using 220 mM acetic acid for 1 hour to induce apoptosis, 6% ethanol for 2 hours, 0.6 mM H₂O₂ for 20 hours, and UV for 50 seconds as stressors to the strains that did not exhibit β -galactosidase activity on the first attempt under normal growth condition. The large-scale transformation was adopted from the protocol for Synthetic Genetic Array (SGA) as performed in yeast by Tong *et al.*, 2001. In this method, the query plasmids were first transformed into MAT α , which was then crossed with the MAT α -based YKO

collection by replication via manual pinning of the YKO on top of the query strain, containing the targeted plasmid in a 384-colony format. After zygote formation, diploids were selected for by taking advantage of selectable markers present in both mated strains (-URA G418). The cells are subsequently forced to go through sporulation, during which the cells were starved for 9 days at 22°C on enriched sporulation media. Spores were then pinned to select for haploids using SD-HAU+Can, and meiotic progeny with both deletion and plasmid were then selected for by pinning on SD/MSG-HAU+G418+Can media. Finally, meiotic progenies were pinned on -URA G418 directly prior to performing colony lift assay.

4.4.6 Large-scale colony lift assay

The MATa meiotic progenies, containing p281-4-IRES, were grown on -URA medium. Containing G418 and 2% galactose for 2 days at 30°C. Fully grown colonies from the resulting array were lifted using nitrocellulose membrane. Membranes were submerged in liquid nitrogen to instantly freeze the colonies and once thawed outside at room temperature, they were submerged in a solution of Z-buffer pH 7.0 containing 1 mg/mL X-gal and 0.27% β -mercaptoethanol. Colonies that remained yellow or turned blue substantially slower compare to majority of the colonies were recorded as positive hits for potential genes encoding proteins that may have ITAF activity for each targeted IRES.

4.4.7 Reverse Transcriptase quantitative PCR (RT-qPCR)

This methodology was used to assess the content of target mRNAs. Total RNA was extracted from wild type and knockout strains of the candidate genes identified using

RNeasy Mini Kit (Qiagen). Total RNA samples were reverse-transcribed into complementary DNA (cDNA), 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). qPCR was carried out using BrightGreen qPCR MasterMix (ABM, Cat. MasterMix-S) and the CFX connect real time system (Bio-Rad®), according to the manufacturer's instructions. CFX software was also used to analyze the data. In this experiment, *PGK1* was used as a constitutive housekeeping gene and related to WT (Samanfar *et al.*, 2014;2017). Each qPCR experiment was repeated at least three times using separate cDNA samples.

4.4.8 Immunoblotting

Western blotting was used to quantify relative protein levels. Total protein was isolated using detergent-free methods as described in (Szymanski *et al.*, 2013). Samples were grown overnight in liquid YPD, pelleted and washed with PBS buffer. Bicinchoninic acid assay (BCA) (ThermoFisher®) was used to quantify total protein concentration according to manufacturer's instructions. 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®) (Shaikho *et al.*, 2016). Proteins were transferred to a nitrocellulose 0.45 µm paper (Bio-Rad®) via a Trans-Blot Semi-Dry Transfer (Bio-Rad®). Mouse monoclonal anti-HIS antibody (Santa Cruz®) was used to detect protein level of each IRES gene fused with 6HIS. Pgk1p was also used as a constitutive housekeeping protein for quantification purposes. Mouse anti-Pgk1 (Abcam®) was used to detect Pgk1p levels (Samanfar *et al.*, 2017). 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®), and each density was normalized to the density formed by Pgk1p control. Each experiment was repeated at least three times using three separate total protein isolates.

4.5 Results and discussion

4.5.1 Modification of p281-4-*URE2* by sub-cloning new IRES forming regions

A previously known plasmid called p281-4-*URE2* (Komar *et al.*, 2003) was used to insert new IRES elements in the place of *URE2* gene. This plasmid forms four strong hairpin loop structures at its mRNA molecule which is designed to inhibit cap-dependent translation of *URE2-β-galactosidase* gene (Fig 4-1). The *URE2* gene is fused with a *β-galactosidase* cassette which is an in vivo colourimetric reporter that allows for the direct detection of *URE2*-IRES-mediated expression to be monitored by *β-galactosidase* enzyme activity on its substrate. In this manner, 5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside (BCIG), also known as X-gal was used for large scale screening which produces a distinct blue colour (Toulouse *et al.*, 2017). In our large-scale screening, X-gal was used to test for the presence of *β-galactosidase* enzyme. X-gal is an analog of lactose, which can be cleaved by the *β-galactosidase* enzyme, which yields galactose and 5-bromo-4-chloro-3-hydroxyindole. 5-bromo-4-chloro-3-hydroxyindole instantly dimerizes and is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo which is an insoluble blue colour product. X-gal molecule is colourless however in the presence of blue-coloured product

which can be used to test for the presence of active β -galactosidase (Sandhu, 2010; Toulouse *et al.*, 2017). By using the yeast YKO collection transformed by the newly constructed plasmids, we could screen for the genes that their absence causes a reduction in the production of β -galactosidase enzyme, under the translational control of the designated IRES element.

The DNA inserts coding for eleven yeast genes known to form IRES structures on their mRNAs were PCR-amplified, using sequence-specific primers. The gDNA of wild type (WT) *S. cerevisiae* was extracted and used as the PCR template. Pre-amplified cDNA from tunicamycin-treated HeLa cells was used as the DNA template to amplify inserts, encoding the two IRES regions of the human p53 gene. Expected amplicon sizes and all primer sequence information are available in Appendix 4.A.

After PCR amplification, both the IRES amplicons and p281-4-*URE2* plasmid were enzymatically digested using *EcoRI* and *SacII* restriction enzymes. To obtain the correct directionality required for cloning, the 5' ends of the inserts required to hold *SacII* recognition sites and the 3' ends require to maintain *EcoRI* sites. Correct digestions were confirmed by agarose gel, ligation was carried out overnight. In order to amplify the constructs, after ligation took place, p281-4-IRES plasmids were transformed into *E. coli* DH5 α competent cells. Colony PCR was used to confirm the new transformed bacterial strains for the presence of the IRES regions on each individual plasmid. All cloned IRES elements were confirmed to be present as determined by colony PCR (fragment sizes are available in Appendix 4.A). The plasmids containing IRES constructs were subsequently extracted from *E. coli* and used to transform WT yeast (MAT α) using the lithium acetate

method (see Materials and Methods).

4.5.2 Evaluation of the new constructs in *S. cerevisiae*

After transformation of MAT α yeast with the newly constructed plasmids, non-quantitative ONPG-based β -galactosidase activity assays were carried out to test for the IRES expression in WT yeast under normal condition. We subsequently continued this experiment under IRES inducing conditions for the IRESs, showing no activity under normal condition. In this technique ortho-nitrophenyl- β -galactoside (ONPG) is cleaved by β -galactosidase enzyme, which leads to the production of o-nitrophenol a bright water-soluble yellow compound that can be quantified at 420 nm by a regular spectrophotometer (Toulouse *et al.*, 2017). By placing the β -galactosidase reporter gene under the control of a particular IRES element. In this experiment, the amount of expression via the designated IRES is directly detectable by the amount of enzyme activity of which results in the production of yellow colour.

Figure 4-1: General schematic diagram of the mRNA transcript produced from p281-4-IRES constructs. The four highly stable inhibitory hairpin loops prevent the ribosome from scanning for the AUG start, via cap structure. β -galactosidase enzyme is expressed under the control of the particular IRES element fused upstream of it.

Many IRES elements can be constitutively translated under normal condition (Komar *et al.*, 2003; Toulouse *et al.*, 2017; Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018), resulting in the production of o-nitrophenol which indicates the expression of β -

galactosidase through the IRES element. Other IRES regions may become active or hyper-active under certain stress conditions (Komar *et al.*, 2003; Spriggs *et al.*, 2008; Schrufer *et al.*, 2010; Toulouse *et al.*, 2017) which suggests that the translation through IRES maybe facultative under those particular conditions. Out of the newly constructed 12 IRES- β -*galactosidase* plasmids, 5 showed positive signal for β -galactosidase activity which indicates constitutive expression under normal condition (Table 4.1). These IRESs were those belonging to *FLO8*, *GPR1*, *HAP4*, *YAP1*, and *NCE102*, all of them native to *S. cerevisiae*. The remaining yeast IRESs and p532-IRES were further tested under stress conditions known to induce IRES-mediated translation (Reineke *et al.*, 2011; Samanfar *et al.*, 2017; Moteshareie *et al.*, 2017) (Table 4-1). These treatments included the followings; 6% ethanol (2 hours) and 220 mM acetic acid (1 hour). Ethanol is a known inhibitor of eIF2A, which is a factor that is suggested to downregulate IRES-mediated translation of *URE2*-IRES when the cell is under stable conditions (Reineke *et al.*, 2011). The presence of 6% ethanol has previously shown to downregulate the expression of eIF2A, therefore it was expected to upregulate the internal initiation (Reineke *et al.*, 2011). A typical physiological stress response to apoptosis is the increased use of IRES-mediated translation (King *et al.*, 2010). The stress from the exposure to acetic acid can lead to the induction of apoptosis (Samanfar *et al.*, 2017) therefore it was thought to upregulate the IRES-mediated initiation of translation. Unfortunately, neither of these conditions yielded any positive signal from IRES-mediated translation of the remaining IRES elements to be confirmed (Table 1).

In peruse to further test the activity of the unconfirmed IRESs, *TIF4631*-IRES and both p53 IRESs were further tested after exposing cells to reactive oxygen species (ROS),

which typically leads to the induction of apoptosis (Perrone *et. al.*, 2008). In this case, cells were treated with 0.6 mM hydrogen peroxide (H₂O₂) for 20 hours. Once again, this did not yield any appreciable IRES-mediated activity. The final test for the same three IRESs as above was carried out using UV radiation for 50 seconds, a condition reported to induce DNA damage culminating in apoptosis (Carmona-Gutierrez *et. al.*, 2010). Unfortunately, our quest to induce the rest of the constructed IRESs, even with this last treatment, yielded no detectable IRES-mediated expression either (Table 4-1).

Table 4-1. Results of the non-quantitative *β-galactosidase* activity test for IRES activity, performed on MAT α strains transformed with the new p281-4-IRES constructs, under both normal and IRES-inducing stress conditions. IRES elements that showed expression are ranked in order from fastest to slowest detection of activity. “No signal” indicates that no activity was detected. “Not tested” shows that the IRES in question was not tested under the given conditions indicated in the table.

Condition		No treatment	60% EtOH (2 hrs)	220 mM HOAc (1 hrs)	0.6 mM H ₂ O ₂ (20 hrs)	UV (50 s)
IRES element	<i>BOI1</i>	No signal	No signal	No signal	Not tested	Not tested
	<i>FLO8</i>	First signal	Not tested	Not tested	Not tested	Not tested
	<i>SPT15</i>	No signal	No signal	No signal	Not tested	Not tested
	<i>GPR1</i>	Fifth signal	Not tested	Not tested	Not tested	Not tested
	<i>TIF4631</i>	No signal	No signal	No signal	No signal	No signal
	<i>HAP4</i>	Third signal	Not tested	Not tested	Not tested	Not tested
	<i>YAP1</i>	Second signal	Not tested	Not tested	Not tested	Not tested
	<i>MSN1</i>	No signal	No signal	No signal	Not tested	Not tested
	<i>YMR181C</i>	No signal	No signal	No signal	Not tested	Not tested
	<i>NCE102</i>	Fourth signal	Not tested	Not tested	Not tested	Not tested
	p53 ¹ (FL-P53)	No signal	Not tested	Not tested	No signal	No signal
	p53 ² (Δ N-P53)	No signal	No signal	No signal	No signal	No signal

To confirm whether the lack of activity was not due to incorrect insertion or mutations, all constructed plasmids were sent for DNA sequencing. Sequencing analysis confirmed the correct sequence of the following IRESs *FLO8*, *HAP4*, *YAP1*, *MSN1*, *NCE102*, and p532 IRES elements correctly inserted into p281-4 in the correct place of *URE2*-IRES (these results are available in Appendix 4.B). Although previously

confirming the presence of the inserts in the plasmids for *BOII*, *GPR1*, *SPT15*, and *TIF4631* IRESs via colony PCR supports the presence of their IRES regions, sequencing results for plasmids with forming regions came back as partially sequenced. Possibly due to excessive sample contamination by plasmid extraction kit components (e.g. salt or ethanol contaminations) as well as the degradation of template plasmid or sequence-specific primers (Green and Sambrook, 2016).

This suggest that the strains may have not been successfully transformed and thus do not carry the plasmid. However, this is highly unlikely because of the presence of a *URA3* selectable marker on the p281-4-IRES plasmids, which allows only for those colonies that carry the plasmid to survive on an appropriate selective medium (SC -URA). The lack of activity in the unconfirmed IRES elements could also be explained by incorrect identification of IRES structure in the first place. Although all of our targeted IRES elements can be found either indicated in the online database IRESite or suggested in the literature, confirmation of the existence of these internal structures has only taken place utilizing activity assays or other indirect techniques (Zhou *et. al.*, 2001; Xia and Holcik, 2004). In support of this notion, there has been a long ongoing debate as to whether some of these genes in fact form IRES structures or not (Zhou *et. al.*, 2001; Mauro *et. al.*, 2004). However, with regards to the lack of activity unveiled by the two native human p53 IRESs, it is very well possible that they indeed require certain human ITAFs to fully function which indicate that cellular IRES-mediated translation is a rather selective process, especially in terms of the host organism. These specific factors are most likely not available when using heterologous host organisms such as yeast. In addition, previous studies of working with exogenous proteins in yeast system have suggested that

eukaryote-specific extensions of conserved ribosomal proteins of the small subunit may facilitate eukaryote-specific interactions in the initiation phase and specifically IRES-mediated initiation phase of translation which may be the reason why human IRES may not be functional in yeast (Ghosh, and Komar, 2015). It is equally important to note that due to the large size of the entire genes, we solely cloned the IRES region of each gene that we were interested in to study. Therefore, it is likely that the full coding region may have been required for these IRES elements to express any activity.

4.5.3 Candidate gene identification via large-scale screening

Based on their activity resulted from our *β-galactosidase* assay and support from previous studies, *URE2*, *FLO8*, *GPR1*, *YAP1*, and *NCE102* were selected to identify their associated ITAFs by screening yeast non-essential deletion library YKO (MATa). Consequently, the newly constructed plasmids, containing 4 new IRES elements and original *URE2*-IRES were transferred into yeast MATa WT strain and subsequently crossed with the YKO (MATa), containing more than 4300 individual non-essential gene knockouts. For this experiment, a large-scale transformation (LST) was used which is heavily based on the protocol for synthetic dosage lethality or SDL (Measday and Hieter, 2002). After several selection steps which resulted in an array of MATa meiotic progenies harboring our IRES containing plasmids and a gene deletion. These mutant arrays were plated on SC -URA media containing galactose instead of glucose in order to induce the GAL1/10 promoter. This specific medium was also supplemented with a sub-inhibitory concentration of the antibiotic geneticin (G418) in preparation for the next experiment which is known as colony lift assay. This technique involves the lifting of MATa deletion

colonies containing a query plasmid, which is subsequent lysed and exposed to a solution containing the lactose analogue X-gal. If the β -galactosidase enzyme is present as a result of active expression of *β -galactosidase* locus under IRES-mediated translational control, X-gal substrate will be cleaved by the enzyme to yield 5-bromo-4-chloro-3-hydroxyindole (a blue-green compound). When a particular strain of yeast is missing a gene involved in regulation of IRES-mediated translation for a specific IRES, a decrease in *β -galactosidase* expression is very likely. Consequently, the colony in question should remain with no sign of blue colour for a longer period (Fig 4-1). Therefore, as a result of colony lift assay, colonies that remained yellow for longer in comparison to the majority of other colonies (which turned blue faster) were selected as positive hits (Fig 4-1).

Figure 4-2: Demonstration of a colony lift assay on a nitrocellulose membrane. The cells on the membrane was lysed in liquid nitrogen and then incubated in a solution containing the substrate X-gal, which yields a blue coloured compound upon cleavage by the β -galactosidase enzyme. Colonies lacking genes that may be involved in IRES-mediated translation will remain with no apparent blue colour, since they will exhibit less *β -galactosidase* activity. Examples are circled in red.

After analysing our results from colony lift assay, a list of genes for each individual IRES element was organised with all the genes that found to be positive hits in at least two of our three colony lift repeats. In our total screening, a total number of 171 genes were identified, screening results performed with *URE2*-IRES, showed 41 positive

hit genes, for *FLO8*-IRES, *GPR1*-IRES, *YAP1*-IRES and *NCE102*-IRES, we obtained 38, 59, 48, and 39 genes respectively (please refer to Appendix 4-3 for the full list of genes identified for each IRES element). From our final candidate lists, another list of 33 candidates was developed for each IRES from the genes found in common between at least three IRES elements (Table 4-2). These 33 genes were considered to be our general ITAF candidate genes.

Table 4-2. Lists of genes recorded as positive hits for 3 or more IRES elements (highlighted in purple). Highlighted genes in orange are part of a shortlist of genes common between 4 or more IRES structures which were selected as candidate genes in this study for further investigation.

<i>FLO8</i> -IRES	<i>GAL1</i>	<i>AVT2</i>	<i>YNR018W</i>	<i>YAP1</i> -IRES	<i>RMD11</i>	<i>GAL1</i>	<i>SOH1</i>	<i>URE2</i> -IRES	<i>GSH2</i>
	<i>HAP3</i>	<i>SLM3</i>	<i>GSH2</i>		<i>YMR074C</i>	<i>GSH2</i>	<i>THP2</i>		<i>FET3</i>
	<i>RMD11</i>	<i>DBF2</i>	<i>BTS1</i>		<i>NCE102</i>	<i>CSG2</i>	<i>COX5A</i>		<i>THP2</i>
	<i>COX5A</i>	<i>COX5A</i>	<i>YOR246C</i>		<i>SOH1</i>	<i>SLM3</i>	<i>MKS1</i>		<i>SOH1</i>
	<i>MKS1</i>	<i>MKS1</i>	<i>NCE102</i>		<i>THP2</i>	<i>MFT1</i>	<i>LIP5</i>		<i>COX5A</i>
	<i>IES2</i>	<i>MEP3</i>	<i>ASN1</i>		<i>IES2</i>	<i>FET3</i>			<i>BTS1</i>
	<i>YNR018W</i>	<i>ILAP3</i>	<i>CDA2</i>		<i>ILAP3</i>	<i>BTS1</i>			<i>ILAP3</i>
	<i>LIP5</i>	<i>CSG2</i>			<i>RTG3</i>	<i>CDA2</i>			<i>NCE102</i>
	<i>YOR246C</i>	<i>RPL19A</i>			<i>BCS1</i>	<i>HAP3</i>			<i>SLM3</i>
	<i>YGR273C</i>	<i>RTG3</i>			<i>COX5A</i>	<i>RPL19A</i>			<i>COX5A</i>
	<i>AXL1</i>	<i>GAL3</i>			<i>YNR018W</i>	<i>BCS1</i>			<i>LIP5</i>
	<i>YMR074C</i>	<i>PEX7</i>			<i>YGR273C</i>	<i>GAL3</i>			<i>YGR273C</i>
	<i>KGD2</i>	<i>RTT103</i>			<i>AXL1</i>	<i>PEX7</i>			<i>RTG3</i>
	<i>NCE102</i>	<i>THP2</i>			<i>ASN1</i>	<i>KGD2</i>			
	<i>AVT2</i>	<i>MFT1</i>			<i>MEP3</i>	<i>RTT103</i>			
		<i>FET3</i>			<i>CDA2</i>	<i>DBF2</i>			

Five candidates (*HAP3*, *COX5A*, *NCE102*, *CDA2*, and *THP2*) from our short list were selected for follow up experiments. Out of these five genes, *HAP3*, *COX5A* were selected as positive hits for all five tested IRES elements, suggesting they may potentially hold more general ITAF activities. Whereas, *NCE102*, *CDA2*, and *THP2* were positive hits for four IRESs out of five (Fig 4-2). Other general ITAF candidates, identified in our screening process, should be investigated in future experiments in the same manner as the selected genes for this project. We aimed to observe the comparative analysis of IRES-mediated translation of the five tested IRES elements in the presence and absence of our

potential nonspecific ITAF candidates. In order to support the ITAF activity of our five candidate genes, we conducted complementary gene expression analysis for the five tested IRES elements in the absence of our five ITAF candidates.

Moreover, it is critical to examine the expression of the tested IRES elements at the transcriptional levels in the absence or presence of our ITAF candidates. It is possible that some of our candidate genes may play a role in the transcriptional regulation of the tested genes containing IRES forming region, rather than having actual ITAF activity. Consequently, such an experiment with the objective of evaluating relative levels of mRNA contents of IRES containing genes was carried out, using Reverse Transcriptase quantitative PCR (RT-qPCR) experiments in the absence or presence of the candidate genes.

4.5.4 Quantification of IRES gene mRNA content via RT-qPCR

In order to entirely attribute the reductions in *β-galactosidase* activity to a decrease in IRES-mediated translation, rather than regulation of transcriptional activity in the observed phenotypes (lack of blue colour production), RT-qPCR was performed. To this end, we used total RNA extracts from WT and YKO MATa strains lacking each of the candidate genes and subsequently converted these the total RNA into cDNA. The cDNA was then used as templates in the RT-qPCR experiments, which involved amplification of the transcripts of each IRES gene using sequence-specific primers (Appendix 4-1). Our specific objective in this experiment was to examine whether there were no reductions in the level of normalized mRNA contents of the targeted IRES genes in the deletion strains of our candidate genes in comparison to WT strain. A reduction in mRNA content of the targeted IRES gens would suggest that the positive hit in our

screening is partially due to a downregulation at transcriptional level. To normalize our qPCR results, the yeast housekeeping gene *PGKI* can be used as an internal control.

The normalized mRNA content of the five targeted IRES genes showed no significant reductions in the absence of our five nonspecific ITAF candidates (Fig 4-3). In the absence of *CDA2* and *THP2*, the mRNA levels remained relatively constant with respect to their WT levels. These results suggest that *CDA2* and *THP2* may not be involved in the transcriptional regulation of any of the targeted IRES containing genes, instead may regulate them for IRES-mediated translation. However, when *HAP3*, *COX5A*, or *NCE102* were deleted, no reduction was observed with respect to WT in the mRNA content of targeted IRES genes. Instead, the amount of mRNA increased by around 15% for *NCE102* in the absence of *COX5A* and by 30-40% for *YAP1* in the absence of *HAP3*, *COX5A* and *THP2* (Fig 4-3).

Figure 4-3: The relative mRNA levels of the targeted IRES genes quantified by normalizing the mRNA content of the mutant strains to those in the wild type. The house keeping gene *PGKI* was used as an internal control. Deletion of the ITAF candidates had no significant reduction on non-of the normalized mRNA contents of our targeted IRES containing genes. Each experiment was repeated at least three times. Error bars represent standard deviations.

The elevated level of *YAPI* mRNA suggests a potential downregulatory role for the deleted genes at the transcriptional level of *YAPI*. However, in order to further support the transcriptional role for these genes, complementary experiments are required. It is important to note that these outcomes certainly do not disqualify these three candidate genes from potentially being involved in regulation of IRES-mediated translation of *YAPI* upon which they may also be involved in transcription-downregulation. Therefore, it seems very likely that both cellular functions coexist, possibly each one may become activated at a different time. Up to date, majority of discovered human ITAFs are commonly known to have RNA-binding domain hence it may also possible for some of undiscovered ITAFs to have the ability to bind to DNA as well (Martinez-Salas *et. al.*, 2013). This protein family is broadly known as DNA- and RNA-binding proteins or DRBPs (Hudson and Ortlund, 2014). Even though our RT-qPCR trials were conducted in triplicates, another possibility is that these increases can be a result of random experimental errors.

Over all, our RT-qPCR analysis suggests that although there has been some upregulation at the mRNA levels of *YAPI* in the absence of *HAP3*, *COX5A* and *THP2*, in the absence of non-of our ITAF candidates, the targeted IRES genes were downregulated at the transcriptional levels, which supports the role of our ITAF candidates. After this validation, to directly quantify the involvement of our ITAF candidates in IRES-mediated translation of each targeted IRES, we conducted an ONPG based experiment to quantify the β -galactosidase under translational control of the targeted IRES elements.

4.5.5 Validation and quantification of candidate involvement in IRES

The relevant deletion strains of the targeted ITAF candidates were subjected to a liquid ONPG-based quantitative *β-galactosidase* assay to validate and quantify the reductions in IRES-mediated translation of *β-galactosidase* enzyme observed in our high-throughput screening analysis. The same methodology as the *β-galactosidase* activity assays used to validate the IRES activity after yeast MAT α transformation, prior to the large-scale transformation (Table 4.1). However, in this experiment, the assayed yeast strains each have a candidate gene deleted in addition to carrying p281-4-IRES plasmids in addition to quantification of the produced yellow colour which is directly under control of IRES-mediated translation of the *β-galactosidase* mRNA.

The quantification of *β-galactosidase* activities under translational control of all targeted IRES elements were reduced in the absence of the all targeted ITAF candidates. These results are ranging from 30% to 100% reduction, relative to WT levels (Fig 4-4) which also supported the integrity of our high-throughput screening analysis. Although all strains showed uniformly strong reductions in *β-galactosidase* activity with respect to WT strain, the only gene deletion that did not show a strong reduction in IRES-mediated translation of only *GPR1* mRNA (Fig 4-4). The strongest influence out of all targeted ITAF candidates was also imposed on IRES-mediated translation of *GPR1* mRNA in the absence of *HAP3*, *NCE102*, *CDA2* or *THP2* resulting in expression of effectively no detectable *β-galactosidase* activity. Once again, this indicates a strong regulatory role for these four potential ITAFs towards specifically IRES elements of *GPR1*. Equally, the most promising nonspecific potential ITAF candidate, seems to be *THP2*. Deletion of *THP2* showed a significant reduction in the production of *β-galactosidase* enzyme under

translational control of effectively all five targeted IRES elements (Fig 4-4). In addition, given the evidence that *THP2* was previously identified as a positive hit in our previous screening experiments for IRES elements of two heat shock proteins namely, *HSP82* and *HSC82* (Samanfar, 2014). *HAP3* also demonstrated a significant reduction in β -galactosidase activity under translational control of all five targeted IRES elements (Fig 4-4) which potentially is an ideal ITAF candidate for follow up experiments.

Figure 4-4: Effect of gene deletion on translation and transcription of β -galactosidase mRNA. The relative β -galactosidase activity is determined by normalizing the activity of the mutant strains to that of the WT strain. Bars represent β -galactosidase activity under the IRES-mediated translational control of the targeted IRESs respectively signified by different colour. Each experiment was repeated at least three times. Error bars represent standard deviations. * Indicates statistically significant differences (t-test) between WT cells and mutant cells ($p < 0.005$).

Many years of investigation into cellular and viral IRES-mediated translation, strongly suggests that under physiological stress conditions such as endoplasmic reticulum (ER) stress, hypoxia, nutrient limitation, mitosis and cell differentiation that canonical cap-dependent initiation of translation is inhibited, IRES-mediated translation takes over and mRNAs containing IRES become more competitive for the available pool of translational components and ITAFs (Komar, and Hatzoglou, 2005; Lewis, and Holcik, 2008; Lacerda *et al.*, 2017). Many of the cellular mRNAs that contain IRES elements

encode proteins that are essential for the protection of cells from stress or, alternatively, induction of apoptosis (Mokrejš *et al.*, 2009; Samanfar *et al.*, 2017). Since under these stress conditions, critical ITAFs must be present for proper IRES-mediated initiation of translation, there should exist IRES genes that at the same time would have ITAF activity. Our results demonstrated that *NCE102* is a potential nonspecific ITAF which itself is translated through IRES-mediated translation, perhaps by using other ITAF candidates (Table 4-2). It is noteworthy that Nce102p is a transmembrane protein that has been linked to eisosome formation (Kim *et al.*, 2016; Gournas *et al.*, 2018). This, in contrast, challenges that how Nce102p may be able to also exert its function as an ITAF, given the fact that transmembrane proteins are mainly confined to two-dimensional movements across the cell membrane (Jacobson *et al.*, 1987). However, it is also very likely that the IRES elements that require Nce102p are recruited or localized to the plasma membrane, where Nce102p is able to bind the IRES and active IRES-mediated initiation of translation. This can be supported by the fact that translation occurs in the cytoplasm with an extensive and highly fluid environment (Crick, 1958). It may also be possible that Nce102p can exert its ITAF activity prior to translocation to the membrane. To this end, the *NCE102* gene requires further investigation into its role as an ITAF. The fact that *NCE102* mRNA contains IRES element and at the same time it also appears to have a nonspecific ITAF activity, strongly suggest that cellular IRES-mediated translation plays a key role in the fate of a cell and cellular decision making under a variety of stress conditions (e.g. Samanfar *et al.*, 2017; Moteshareie *et al.*, 2018). In these stress conditions that inhibit cap-dependent translation, different cellular IRESs impose various responses by regulating the expression of cellular ITAFs. They are responsible for sensing changes

in cellular metabolism and modulate their expression accordingly which influence IRES activity (Komar and Hatzoglou, 2005; Spriggs *et al.*, 2005; Lewis, and Holcik, 2008).

Over all, this experiment demonstrated that in the absence of the examined identified nonspecific ITAFs the normalized β -galactosidase activity is reduced, underlying the decrease in *β -galactosidase* reporter expression under the translational control of each targeted yeast IRES element. The main weakness of quantifying IRES-mediated translation through this method is that the expression of the targeted IRES-reporters is via a plasmid. In this experiment, β -galactosidase enzyme, under translational control of IRES, is produced from p281-4-IRES plasmids which is under the control of a galactose-inducible promoter and may take place in a form of overexpression. This can lead to aberrant results given that the levels of protein present are not native (Komar *et al.*, 2003; Prelich, 2012). In addition, the native protein expression of our targeted IRES genes is under translational controls of both canonical cap-dependent and IRES cap-independent initiations hence it may be difficult to allocate any potential native reduction in the activity of each IRES as a result of gene deletion. However, the plasmid-based overexpression may be beneficial to produce enough reporter activity for proper signal detection. Therefore, plasmid-based *β -galactosidase* reporter assay may be considered more circumstantial evidence and should be paired with other experiments such as analyses of endogenous gene expression such as western blotting and polyribosome profiling. For the time constrains in this study, as an example, we continued our analysis by investigating the native protein expression of all five targeted IRES elements in the absence of *HAP3* compare to WT strain. This experiment will more directly establish a link between *HAP3* as a potential nonspecific ITAF for all five targeted IRES elements.

4.5.6 Reduction in the protein contents of all five IRES elements in the absence of *HAP3*

Subsequently, we investigated the level of endogenous proteins produced via all five targeted IRES elements in the presence and absence of *HAP3* in the cells by western blot analysis. This was accomplished by fusing our five IRES genes individually to six histidine (6HIS) at the genomic level. Our analyses showed a reduction in the endogenously-expressed our targeted IRES-6HIS fusion protein levels in the absence of *HAP3* (Fig 4-5). When *HAP3* was deleted, protein levels of all our five targeted IRES elements were reduced ranging from approximately 50% to 70%, compared to the WT cells (Fig 4-5). This suggests that *HAP3* plays an important role in regulating the expression of our targeted IRES genes. In addition, deletion of *HAP3* did not change the protein levels of Pgc1p, which was used as an internal control.

Figure 4-5: Western blot followed by densitometry analysis to measure each IRES-6HIS levels in *hap3Δ* strain. Values are normalized to that for Pgc1p, used as an internal control and related to the values for WT strain. Cells are grown under standard laboratory conditions. Each experiment was repeated at least three times. Error bars represent standard deviations. * Indicates statistically significant differences (t-test) between WT cells and mutant cells ($p < 0.005$).

The translation of all targeted IRES genes are under translational control of both cap-dependent and cap-independent initiation of protein synthesis. Therefore, our immunoblotting analysis cannot definitively attribute the observed reductions to the absence of *HAP3*. However, our previous results from β -galactosidase assay analysis supported no role for *HAP3* in the regulation of general cap-dependent translation of β -galactosidase gene. This and the fact that *HAP3* reduced β -galactosidase activity under translational control of the five targeted IRES elements, suggest that *HAP3* may only be involved in IRES mediated translation of the targeted IRES elements. Overall, more evidence is required to conclusively support a direct connection between all examined nonspecific ITAF candidates and IRES-mediated translation of the targeted IRES elements.

4.6 Conclusion

We have successfully amplified 10 yeast IRES forming regions along with two IRES regions found in human p53 gene by using sequence-specific PCR reactions. This resulted in amplicons of the correct sizes in all cases which was visualized on agarose gel. The amplicons then inserted into p281-4-*URE2* being replaced with *URE2* through restriction digest and ligation. After construction of new plasmids and transformation of *E. coli* with them. Since these IRES forming regions do not exist in the genome of *E. coli*, the plasmids were confirmed to contain the targeted IRES elements of the correct size by using colony PCR. After cloning in *E. coli*, the plasmids were amplified and extracted, and subsequently, they were transformed into yeast MAT α for further confirmation of IRES activity. Out of 12 transformed strains five were shown to exhibit constant heavy

signal from β -galactosidase activity under translational control of the inserted five IRES regions. Of these, four IRESs along with the original *URE2*-IRES were selected for follow up experiments. The remaining seven transformed yeast strains did not exhibit detectable signal from β -galactosidase activity under translational control of the inserted each IRES region of these seven IRES regions, even after a series of IRES-inducing treatments. In addition to other conformational experiments, sequencing results also revealed the correct sequence of 6 of the 10 cloned IRES elements that were sent for sequencing, while the other 4 were considered to be inherently facultative in expression (at least under the tested experimental conditions). Please see Appendix 4.B for the full sequencing results.

After confirmation of the correct and active constructs, we performed large-scale screening with five IRES elements through which we identified a total of 142 novel genes with specific or nonspecific potential ITAF activity. A total of 23 candidate genes with a potential role in non-specifically regulating the IRES-mediated translation of two or more IRES elements as activator ITAFs were identified. Out of these 23 candidate genes, *HAP3*, *COX5A*, *CDA2*, *THP2*, and *NCE102* were selected for further investigations. Our RT-qPCR analysis suggested that in the absence of none of the candidate genes, transcription of any of the targeted IRES genes were downregulated which directly connects the observed β -galactosidase activity to translation of the targeted IRES genes. ONPG-based β -galactosidase assay also quantified the involvement of the five identified candidate genes in the expression of *β -galactosidase* gene under translational control of the targeted IRES elements. Our ONPG-based β -galactosidase also suggested a significant role for all five tested candidate genes for almost all five targeted IRES elements.

Western blot analysis also revealed the level of endogenous proteins produced via all five targeted IRES elements in the absence of *HAP3* in the cells compared to WT cells which showed a significant reduction in the expression of all five targeted IRES elements in the absence of *HAP3*. Lastly, our findings strongly support the validity of our hypothesis and the integrity of our screening process, which establishes that the identified candidate genes may very well play a significant role in nonspecific IRES-mediated translation of almost all of our targeted IRES elements.

4.7 Future directions

Future follow up experiments are required to unequivocally establish the role of these candidate genes as nonspecific ITAFs in a eukaryotic context, using yeast. Western blot experiments should be carried out to analyze the involvement of all identified candidate genes on the relative endogenous levels of IRES-mediated translation of *FLO8*, *GPR1*, *YAP1*, *NCE102* and *URE2* with respect to wild type. Potential results from this experiment can verify the regulatory functions of these genes in IRES-mediated translation. These results can then be paired with polysome profiling in the absence of the identified potential ITAFs to uncover more about the translational nature of these factors.

In parallel, reintroduction of the candidate genes into the knockout strains used for β -galactosidase activity assays, in case of compensation in the reduced β -galactosidase activity, would also provide a compelling support for the observed phenotype not to be an artefactual in the absence of the identified candidate genes. Given the high rate of false positives and negatives which techniques such as the colony lift assay in large-scale screen yields, it may be beneficial to investigate genes from overrepresented families in the lists of positive hits obtained from the large-scale screening. This should include

genes found through any of the three colony lift replicates for each IRES element. In this way, it may be possible to find potential candidates which did not necessarily exhibit a promising phenotype throughout the colony lifts. The presence of several genes from the same family may indicate some kind of relation between the process represented by said family and that of IRES-mediated translation. Although no gene family stands out from our screen's results, this may no longer be the case if the screens were to be repeated.

Investigation into the interaction networks that the discovered novel ITAF genes form, can also be analyzed to identify related genes and establish whether the identified interacting partners also play translational roles. If this is the case, this would further link the ITAF genes to translation regulation. Finally, the interactions between our four target IRES elements and the potential ITAFs encoded by the candidate genes we identified should be studied in the context of biomolecular interaction studies. *In-vitro* techniques such as surface plasmon resonance or more *in vivo* alternatives such as RNA-based pull-down experiments can be performed in the future to characterize the candidates' regulatory role in a more direct physical manner. Furthermore, unconfirmed newly constructed plasmids for the IRES activity can also be tested under additional IRES-inducing treatments such as heat shock (Kim and Jang, 2002; Samanfar *et al.*, 2017). In case any of other IRESs are confirmed to be functional under the new experimental conditions, we can use them for large-scale screening under these conditions. In addition, we can also screen for the genes that their absence can upregulate IRES-mediated translation. Over all, we believe that these results may lead to further work aiming to elucidate distinct IRES mechanisms shared between different cellular IRESs.

5 Chapter 5: Manganese-induced cellular disturbance in the baker's yeast, *Saccharomyces cerevisiae* with putative implications for neuronal dysfunction provoked by this metal

5.1 Abstract

Manganese (Mn) is an essential element; but in humans, chronic and/or acute exposure to this metal can lead to neurotoxicity and neurodegenerative disorders including Parkinsonism and Parkinson's Disease by unclear mechanisms. To better understand the effects of Mn^{2+} exposure on the biology of a cell we exposed the yeast, *Saccharomyces cerevisiae* non-essential gene deletion collection to a sub-inhibitory concentration of Mn and followed by targeted functional analyses. This screen produced a set of 68 sensitive gene deletion mutants and functional enrichment analysis of these genes revealed an enrichment for genes associated with the process of protein biosynthesis. Our follow-up investigations uncovered that Mn reduced the total levels of yeast rRNA molecules in a dose dependent manner and reduced the expression of a β -galactosidase reporter gene. This was subsequently supported by analysis of ribosome profiles in the presence or absence of Mn. The results suggested that Mn-induced toxicity was associated with a reduction in active polysomes. Lastly, using the Comparative Toxicogenomic Database we revealed that Mn shared certain similarities in toxicological mechanism with neurodegenerative disorders including Alzheimer's, Parkinson's and Huntington's diseases. Altogether, these findings contribute to the current understanding of the mechanism of Mn^{2+} -triggered cytotoxicity.

5-2 Introduction

Trace elements play an important role in the balance of life on our planet. The ability of the cell to utilize these elements depends strongly on their concentration, chemical speciation and fractionation (Templeton *et al.*, 2000). Manganese (Mn) is the twelfth most abundant element in the earth's crust. Natural levels of Mn range from 1 to 200 µg/L in fresh water and 410 to 6700 mg/kg (dry weight) in sediment (Howe *et al.*, 2004). In aquatic environments, Mn^{2+} is the most dominant and stable water-soluble species when the pH and redox potential are kept low (Howe *et al.*, 2004). The Mn^{3+} is soluble only in complex form and the Mn^{4+} has very limited solubility (Klewicki and Morgan, 1998). Different Mn species can interconvert via oxidative or reductive processes depending on the redox environment (Klewicki and Morgan, 1998; Templeton *et al.*, 2000). Additionally, it is known that the Mn is a vital trace mineral in nutrition (Luo *et al.*, 2007). Due to geogenic reasons and anthropogenic activities such as mining, local levels of Mn can be significantly higher in certain areas (Hafeman *et al.*, 2007; Hernández *et al.*, 2009). Higher levels of Mn can have negative consequences for environmental health.

Epidemiological and toxicological studies suggest that at certain concentrations, this metal can be detrimental to specific biological processes and beneficial to the others. This is influenced by developmental stage, disease state (Peres *et al.*, 2016; Pfalzer and Bowman, 2017) cell type (Hernández *et al.*, 2011), and/or the organism itself. Studies with different cell models from different organs including the liver, kidney and brain, suggest that neurotoxicity is the principal effect of this metal (Hernández *et al.*, 2011). However, the mechanisms behind this effect is unclear and is the subject of current

studies (Tarale *et al.*, 2016). Some investigations in different biological models have provided strong evidence that Mn can disturb the vital flow of genetic information from DNA to RNA to protein (Putrament *et al.*, 1975; Dambach *et al.*, 2015).

The addition of MnCl_2 (5 mM) to highly purified membrane fractions of rat-liver caused a 30% increase in the polysome-binding capacity of stripped rough endoplasmic reticulum (ER) membranes, whereas four- to five-fold increases were observed with smooth ER membranes (Donaldson *et al.*, 1981). Previous studies in yeast suggested that the Mn^{2+} inhibits protein synthesis and nuclear DNA replication as well as mitochondrial erythromycin-resistant mutations, possibly through interference with mitochondrial DNA polymerase(s) (Putrament *et al.*, 1975; Supek *et al.*, 1977). Overwhelming evidence indicates that when stressed or undergoing environmental adaptation cells accumulate non-synonymous mutation (Pan, 2013). Several studies about Mn-trafficking suggested that Mn-induced Parkinsonism can be as a result of mutations in genes *SLC30A10*, *ATP13A2* and *ZnT10* (Leyva-Illades *et al.*, 2014; Nishit *et al.*, 2016). Also His $\rightarrow$ Asn reversion mutant in *hZnT1* conferred Mn transport activity and loss of zinc transport activity (Nishit *et al.*, 2016). *ZnT10* codes for a protein that is localized to the plasma membrane and is involved in zinc homeostasis in subcellular localization (Nishit *et al.*, 2016), while *SLC30A10* codes for a cell surface-localized Mn efflux transporter that reduces cellular Mn levels and protects against Mn-induced toxicity in neurons and worms (Leyva-Illades *et al.*, 2014). The synthesis of these proteins is controlled by the ribosome in connection with the ER (Wang *et al.*, 2015). ER is a large, continuous membrane-bound organelle with distinct domains and numerous membrane contact sites at the plasma membrane, Golgi, mitochondria, and other cellular components including

the SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) complex that participates in ER formation, fusion and function (English and Voeltz, 2013). Protein synthesis is a crucial process for all cells. Due to its central importance to cell survival and its energy requirements, protein synthesis is firmly regulated and strongly connected to other cellular processes, including cell cycle and metabolic pathways (Warner, 1999; Thomson, 2013). Also, it is highly conserved through the course of evolution from higher to lower eukaryotes as well as prokaryotes. In line with this, several aggregation-prone yeast proteins have human homologues that are implicated in protein misfolding associated diseases, suggesting that similar mechanisms may apply in both organisms and that yeast can serve as a good model organism to study such processes (Ibstedt *et al.*, 2014). Studies using ribosome profiling and/or polysome profiling as well as gene expression analysis have provided new insights into the identity of new genes that can affect this process as well as mechanism of protein synthesis itself, which is often considered as an endpoint of gene expression pathway (Ingolia, 2014; Chassé *et al.*, 2017).

Compound genome-wide toxicity can be studied using a systems biology approach, which can decipher the roles of individual components of complex biological systems under certain conditions by examining interactions on a global scale. To this end, large-scale chemical-genomic studies using yeast have been employed to identify individual chemical-genetic interactions and used to generate interaction profiles to infer mechanism(s) of action (Burnside *et al.*, 2015; Márquez *et al.*, 2013). A category of these interactions occurs when the deletion of a single gene causes significant sensitivity or resistance to a target compound. Such interactions can suggest a functional relationship

between the deleted gene and cell responses to the target compound. By screening for chemical-genetic interactions across the genome, significant insights into genotoxicity pathways can be drawn. In this context, protein-protein interaction (PPI) networks also provide a useful resource to better understand the mechanism of toxicity. PPIs underlay nearly all biological processes, including cell-to-cell interactions, and metabolic and developmental controls. Based on their structural properties and functional characteristics, PPIs can be transient interactions that generally participate in signalling pathways or interactions that are more lasting that will form stable protein complexes. It has been revealed that over 80% of proteins do not operate alone but in complexes (Bessarabova *et al.*, 2012; Rao *et al.*, 2014). PPIs can be studied *in vitro*, *in vivo*, and *in silico* (Rao *et al.*, 2014).

Connections to environmental toxin exposures for many human diseases have stimulated studying the toxicity of environmental contaminants in different model organisms (McHale *et al.*, 2018; Escher *et al.*, 2017). Despite the evolutionary distance between yeast and humans, the underlying molecular players of numerous important pathways including programmed cell death, cell cycle progression and gene expression are conserved between the two species, allowing the study of neurotoxins by highly developed omics approaches in yeast (Braun *et al.*, 2010). The range of intra-cellular manganese with physiological relevance or toxicity is quite large. Particularly, various studies done in yeast suggest that the concentrations can be between 2–100 nmol of manganese/(10×10^9 cells), or 0.04–2.0 mM manganese (assuming a single yeast cell has a volume of 50 femtoliters), without any impact on cell growth (Reddi *et al.*, 2009). However, at levels below or above this, the manganese induces toxicity

stimulating cellular responses, including upregulating or downregulating cell surface and intra-cellular transport systems (Reddi *et al.*, 2009). Consequently, yeast has been used to study events associated with Mn homeostasis, neurotoxic cell death, and neurodegeneration (Braun *et al.*, 2010; Büttner *et al.*, 2013). Generally, these studies have used Mn concentrations above of 2 mM, but have hardly explored the large network of physiological pathways that involve manganese, and hence the role of Mn in these processes remains unclear. In the current study, we provide evidence that connect Mn toxicity to the gene expression pathway in yeast. Impairment of protein biosynthesis by Mn^{2+} revealed in this study, improves our current understanding of Mn-induced neurotoxicity and neurodegeneration.

5.3 Materials and methods

5.3.1 Manganese sensitivity/resistance screening using yeast gene deletion array.

Approximately 4700 *MATa* haploid yeast, *S. cerevisiae* strains (BY4741, *MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) from the non-essential Gene Deletion Array (yGDA) were manually arrayed onto agar plates as previously described by Alamgir, et al., 2010 in the presence or absence of sub-inhibitory concentration of $MnCl_2$ (1.35 mM). Plates were incubated at 30°C overnight. Finally, digital images of plates were used to analyze the growth of individual colonies, by automatized and visual density comparison between control and their respective Mn treatment through of the available in-house and online software “SGAtools” from the University of Toronto (Wagih *et al.*, 2013). The experiment was repeated five times and each individual strain had at least 3 independent repeats. Those colonies that showed 30% reduction or more in at least two repeats were

considered hits (Memarian *et al.*, 2007; Márquez *et al.*, 2018).

5.3.2 Transcriptomics experiment using q-PCR

The quantitative PCR(q-PCR) Assay have been used for identification of gene deletion in previous studies (Costa *et al.*, 2004; Traverso *et al.*, 2006). Thus; we used this approach to verify our results about gene deletion array. For it; primers of selected genes were synthetized (Table 5-3). To complete the q-PCR experiment; total RNA was isolated of each strain, using Qiagen RNA isolation kit; followed by cDNA construction, using iScript cDNA synthesis kit with SYBR green supermix (Bio-Rad), according to the instructions of the manufacturer. The quantification of mRNA was performed by q-PCR on a Rotor-Gene RG-300 from Corbett research, according to Samanfar *et al.*, 2017.

Table 5-1: Gene for q-PCR assay

#	Gene Symbol	Sequence (5'→3')	Template strand	Length	Start	Stop	Tm	GC (%)	Self complementarity	Self 3' complementarity	
1	OST2	Forward primer	TCCCGCTAAAAACGCATTGA	Plus	20	516602	516621	58.48	45	4	2
		Reverse primer	CAGCA CGT CAT CTG CAG TCT	Minus	20	516803	516784	60.39	55	6	3
		Product length	202								
2	OST6	Forward primer	CGCTGACAACTACCCA CTGT	Plus	20	233594	233613	59.97	55	3	3
		Reverse primer	TGGCAACTCATGCCGTTACT	Minus	20	233692	233673	59.96	50	4	2
		Product length	99								
3	STT3	Forward primer	TTCGGTGACTTCGTGAA GGG	Plus	20	453244	453263	59.97	55	6	2
		Reverse primer	TCAAGGCA GAA AGTCCGACC	Minus	20	453352	453333	59.97	55	5	3
		Product length	109								
4	DSK2	Forward primer	GGACCTAATGCCGGTATGG	Plus	20	819486	819505	59.96	60	6	3
		Reverse primer	TTCGTGTTGGAGCCTTCCTC	Minus	20	819578	819559	59.97	55	3	1
		Product length	93								
5	ARG3	Forward primer	GTTGCTGAGAGAAACGGTGC	Plus	20	269462	269481	59.76	55	5	2
		Reverse primer	GCTTGGCTGTTTCGCA AAT	Minus	20	269588	269569	60.32	50	4	2
		Product length	127								
6	ICL1	Forward primer	ACCCAGCCTTTGGATGAAGG	Plus	20	285532	285551	59.96	55	4	2
		Reverse primer	GTTACAGAGGTGGGACGCAA	Minus	20	285764	285745	59.97	55	3	1
		Product length	233								
7	CLB2	Forward primer	CAGAGACAGACGGTGCA TGT	Plus	20	772641	772660	60.04	55	4	3
		Reverse primer	CAGCTGCTGCACACAATGAG	Minus	20	772871	772852	60.11	55	7	3
		Product length	231								
8	CDC28	Forward primer	GCCAAAGCTTCTCTCAATGGC	Plus	20	560815	560834	60.11	55	6	2
		Reverse primer	GGGTATACCGGAGGAGTTT	Minus	20	560916	560897	59.82	55	4	1
		Product length	102								
9	np114A	Forward primer	TTGGCCGCTATCGTCGAAAT	Plus	20	431999	432018	60.18	50	6	3
		Reverse primer	TTGCCAGCACTTTTCGTAGC	Minus	21	432120	432100	60	47.62	4	2
		Product length	122								
10	np114B	Forward primer	ACCTAAACCCACCGTGGAC	Plus	20	104415	104434	59.89	55	7	3
		Reverse primer	GTTGGCGTCCCTGGAACATA	Minus	20	104492	104473	60.04	55	3	2
		Product length	78								
11	TIF1	Forward primer	ACTGGTAAGACCGGTACCTTT	Plus	22	555194	555215	59.03	45.45	6	2
		Reverse primer	GCTTGAGGAGCCTTGACAGA	Minus	20	555264	555245	59.68	55	4	1
		Product length	71								
12	HYP2	Forward primer	TGAACATGGACGGTGACACT	Plus	20	85983	86002	59.24	50	5	3
		Reverse primer	GCTTCTTCACCCATAGCGGA	Minus	20	86112	86093	59.82	55	3	2
		Product length	130								
13	UFD1	Forward primer	GCGGCGGAAATGGTTTGTGA	Plus	20	589851	589870	59.76	50	3	2
		Reverse primer	ATTTTCCCGCGGAAGTTTGC	Minus	20	589968	589949	60.04	50	3	2
		Product length	118								
14	UFD2	Forward primer	CGGCGAAAGCAATCGTTCAA	Plus	20	121396	121415	60.11	50	4	3
		Reverse primer	GTGCTCTAAGGCTCAACTCT	Minus	20	121511	121492	59.96	55	6	1
		Product length	116								
15	CDC48	Forward primer	CCA GTACCA GGGGGACATA	Plus	20	237886	237905	60.03	60	4	2
		Reverse primer	CAGTGAGGAAAGGCGACCAT	Minus	20	238201	238182	60.04	55	3	2
		Product length	316								
16	UBC11	Forward primer	ATCGTCTACGGGAAACGCAC	Plus	20	958728	958747	60.46	55	5	1
		Reverse primer	AGTAGAAGAGGGTGGTTCG	Minus	20	958827	958808	59.39	55	2	2
		Product length	100								
17	CDC20	Forward primer	TTGCGTCCCCAACAAAGCTA	Plus	20	289873	289892	60.18	50	4	2
		Reverse primer	ATTAAACGGTGGTGCCCAAT	Minus	20	290059	290040	59.96	50	6	2
		Product length	187								
18	MCM2	Forward primer	GTGGCCAATCTTTCGTCTGC	Plus	20	175437	175456	59.83	55	6	2
		Reverse primer	CGCGCTGCTCAAATTATTGCT	Minus	20	175602	175583	59.97	50	7	3
		Product length	166								
19	NSR1	Forward primer	CTAGAGCCTTCTTGGCGTCC	Plus	20	806681	806700	60.18	60	4	2
		Reverse primer	CCGTCCGTATCCCAACACAT	Minus	20	806773	806754	59.82	55	2	2
		Product length	93								
20	NOP1	Forward primer	ATTGCCCAAGGCAAGAAAGT	Plus	20	427853	427872	60.18	50	7	1
		Reverse primer	TCTCTGCCTGGTCTGTGAGA	Minus	20	427980	427961	59.89	55	4	3
		Product length	128								
21	URA2	Forward primer	CAAAATCTGGATGGGCGCTG	Plus	20	166665	166684	59.9	55	6	2
		Reverse primer	ACGGAAGAAAGCAATCGTGA	Minus	20	166793	166774	60.04	50	6	2
		Product length	129								
22	MCM2	Forward primer	GTGGCCAATCTTTCGTCTGC	Plus	20	175437	175456	59.83	55	6	2
		Reverse primer	CGCGCTGCTCAAATTATTGCT	Minus	20	175602	175583	59.97	50	7	3
		Product length	166								

5.3.3 Total rRNA analysis

The yeast wild type strains were preincubated 1h and then grown on YPD media at 30°C to an OD₆₀₀ of 0.8-1.0, in absence or presence of Mn (1.5 mM or 3 mM), for 0.75, 1 and 24 hours respectively. The number of the cells were normalized before extraction of total RNA. Total RNA was isolated of each strain, using Qiagen RNA isolation kit. RNA electrophoresis was carried out in 1X MOPS running buffer using 10X MOPS buffer (40.8g 3-(N-morpholino) Propanesulfonic acid (MOPS); 6.8g Sodium acetate; 3.8g Ethylenediamine tetraacetic acid (EDTA); volume was completed up to 1000ml by addition of ultrapure water treated with diethyl pyrocarbonate (DEPC), and pH was adjusted to 7.0 with sodium hydroxide (NaOH). The concentration of agarose used in the RNA gels was 1.2% (w/v). Samples were prepared in concentration of 80% v/v deionized formamide, heated at 65°C for 5 minutes, then samples were immediately cooled on ice. Before loading the samples on the gel, 1/10th of sample volume 10X RNA loading dye (0.0125g Bromophenol Blue; 10µl 0.5M EDTA; 2.5ml 100% glycerol; 2.5ml DEPC-treated water; mixed by vortexing and autoclaved) was added to the samples at a final concentration of 1X (1µg).

5.3.4 β -Galactosidase expression assays

The efficiencies of induced translation was measured using an inducible β -galactosidase reporter gene in p416 plasmid (Alamgir *et al.*, 2008; 2010). The enzyme, β -galactosidase is a model of intracellular protein synthesis, which can provide a profile of aberrancy in the rate of protein synthesis and an estimate for gene expression. Thus, samples of the plasmid p416 were preincubated 1 hour and then exposed to a crescent toxicological curve of Mn concentration (0.25 – 3 mM) and similarly to other divalent

ions (Ca^{2+} , Mg^{2+} and Zn^{2+} for 0.5, 1.5, 5 and 15 mM as higher concentration for Mg^{2+}) for 3 hours at 30°C, followed of spectrophotometrically determination of β -galactosidase activity⁸². Ion metal concentrations were suggested by previous works (Loukin and Kung, 1995; Liang and Zhou, 2007).

5.3.5 Polysome profile analysis

Polysome profiling allow for monitoring of translation *in vivo* and it was adopted from Esposito, *et al.*, in 2010. The yeast wild type strains were preincubated 1h and then grown on YPD media at 30°C to an OD₆₀₀ of 0.8-1.0, in absence or presence of 3 mM Mn, for 1 hours and 24 hours respectively at 30°C. Immediately before harvest, cycloheximide was added to all samples, to a final concentration of 100 µg/ml, and the culture was incubated again at 30°C for 15 minutes, followed by cold snap in an ice water bath. Cells were harvested, washed with cycloheximide/water solution (100 µg/ml) and centrifuged at 4000 rpm for 4 min at 4°C using a Sorvall SLA-1500 rotor to separate the supernatant. Cell pellets were resuspended in 10 ml of ice-cold lysis buffer A (YA buffer: 10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 30 mM MgCl₂, cycloheximide 50 µg/ml, heparin 200 µg/ml) and centrifuged at 4000 rpm for 4 min at 4°C (Sorvall SS34 rotor) twice. Pellets were resuspended in 750 µl of YA buffer, lysed by vortexing with glass beads, transferred to microtubes, centrifugated at 13000 rpm for 10 minutes at 4°C. Then; the supernatant was preserved to quantitative determination of total RNA, followed by fractionation on 10-50% sucrose gradients containing 50 mM Tris-acetate [pH 7.0], 50 mM NH₄Cl, 12 mM MgCl₂, and 1 mM dithiothreitol. The extract was centrifuged for 2 h at 40,000 rpm using a SW40-Ti rotor in a Beckman LE-80 K at 4°C. The polysome profiles were analyzed via a Biocomp gradient station, the absorbance was recorded at

254 nm using a spectrophotometer (Bio-Rad Econo UV monitor) coupled with the Biocomp station. In this method, free mRNAs from the top fractions were separated from polysome-associated mRNAs from the bottom fractions (Faye *et al.*, 2014).

5.3.6 Protein-Protein interaction (PPI) prediction and gene ontology (GO) analysis

A PPI network can be described as a heterogeneous network of proteins joined by interactions as edges. Studies about protein network and GO enrichment analysis were based on the data from the current project and analyzed using the STRING database (Szklarczyk *et al.*, 2017). Additional GO analysis was conducted at the Comparative Toxicogenomic Database – CTD (Davis *et al.*, 2016) to verify the hypothesis about conserved mode of action of the manganese between yeast and human. Both STRING and CTD database were accessed at January 31st, 2018.

5.3.7 Data analysis

The results were expressed as mean $\pm$ sem of at least three independent experiments. To detect statistically significant differences ANOVA (analysis of variance) followed by Bonferroni's tests was used; additionally, in some case performed t-test analysis between pairs of treatments. Fitting and statistical analyses were performed with GraphPad Prism (GraphPad 4.0 Software Inc, San Diego, CA, USA).

Data Availability: All data generated and/or analyzed during this study are included in this published article and/or its Supplementary material files).

5.4 Results

To study pathways that are influenced by Mn, we screened for gene deletions that cause increased sensitivity to Mn^{2+} using yeast nonessential gene deletion array (yGDA), figure 5-1A. These types of screens can provide a chemical-genomic interaction (CGI) profile for a target toxin and contribute to our knowledge of the cell's stress responses to that toxin. To this end, for sensitivity analysis we screened approximately 4700 gene deletion strains three times, under two conditions (presence and absence of MnCl_2), for a total of approximately 28,000 individual analyses. Sensitivity was investigated by determining the relative colony growth size in the presence/absence of the target compound. In this way, we identified 68 gene deletion mutants with significantly altered growth profiles (Appendix E, table E-1); of which 43 were confirmed to display high sensitivity to a sub-inhibitory concentration of Mn, figure 5-1B. These genes represent a CGI profile for Mn sensitivity. They often represent “double hits” where the gene deletion and Mn treatment target compensating pathways generating an aggravated effect. The hits identified here were then subjected to further analysis.

Figure 5-1: Representative illustration of Mn-induced disruption in yeast gene deletion array, after exposure to MnCl₂ (1.35 mM), for 24 hours. Mutants that showed relative reduction in growth (sensitivity) of 30% or more were selected as hits ($p < 0.05$). Examples of hit stains are indicated using yellow circles).

5.4.1 Functional proteomic and Gene Ontology analysis of sensitive mutants identifies multiple pathways including protein synthesis

To have a comprehensive coverage of the hits identified in our sensitivity screen, the String database was used to expand the obtained CGI profile for Mn on the basis of protein-protein interaction (PPI) data (Szklarczyk *et al.*, 2017). String uses physical interactions and functional associations to study a defined set of proteins and expand it by including associated proteins. In this way the network of functional interactors for Mn was increased to approximately 600 edges ($p\text{-value} < 1.0\text{e-}16$), of which more than 85% are known interactions. For example, approximately 87% of the interactions have been experimentally verified and almost 98% are from curated databases. A schematic representation of these interactors is shown in Figure 5-2.

Figure 5-2: The inferred and enriched PPI network from 43 genes disrupted by Mn in yeast. Analysis performed with String database. Network properties are as follows: The minimum required of interaction score, to be included at the predicted network, was accepted with a threshold on the high confidence equal 0.7; Number of nodes: 143; Number of edges: 594; Expected number of edges: 287; Average (avg) node degree: 8.31; avg. local clustering coefficient: 0.652; PPI enrichment p-value: $< 1.0e-16$. Nodes and edges represent proteins and PPIs, respectively. Red nodes (protein processing in endoplasmic reticulum); blue nodes (metabolic pathways); dark green nodes (N-Glycan biosynthesis); cyan nodes (cell cycle); yellow nodes (Ubiquitin mediated proteolysis); orange nodes (Meiosis – yeast); maroon nodes (DNA replication); purple nodes (amino acid biosynthesis); magenta nodes (arginine and proline metabolism); lime green nodes (Alanine, aspartate and glutamate metabolism). The protein with black point in the center represents MAPK signaling pathway. Proteins that are not connected to at least one partner are not shown.

Enrichment of cellular pathways represented by the expanded list of proteins is shown in Table 5-2. As expected, proteins associated with development and protein metabolism were highly enriched, which is well documented³⁸. In fact, several authors have documented that protein synthesis is firmly regulated and strongly connected to other cellular processes, including cell cycle and metabolic pathways (Warner, 1999; Thomson, 2013). Particularly, the ER has been previously connected to Mn toxicity

(Wang *et al.*, 2015; Rudgalvyte *et al.*, 2016; Cohen *et al.*, 2013). However, a direct connection between protein biosynthesis and Mn toxicity has not been previously reported. This led us to further investigate the influence of Mn on protein biosynthesis.

Table 5-2: Enriched cellular pathways influenced by Mn in yeast.

Cellular Processes	Pathway description	Number of observed genes	FDR
Cell cycle	Cell cycle - yeast	29	8.46E-21
	Meiosis - yeast	21	1.26E-11
	DNA replication	5	4.39E-03
Biosynthesis and Metabolism of Proteins	Protein processing in endoplasmic reticulum	17	1.78E-11
	N-Glycan biosynthesis	9	2.09E-07
	Arginine and proline metabolism	8	2.84E-06
	Ubiquitin mediated proteolysis	7	6.42E-04
	Alanine aspartate and glutamate metabolism	5	4.12E-03
	Biosynthesis of amino acids	8	3.47E-02
Metabolism	Metabolic pathways	36	2.83E-06

In response to the presence of Mn^{2+} we observed that the levels of total RNA molecules were decreased (Fig 5-3A). After treating the cells with 1.5 and 3.0 mM concentrations of Mn for 45 minutes, total levels of rRNA molecules appeared to be reduced in a dose-dependent manner. We repeated this experiment by increasing the duration of Mn treatment to 3 and 24 hours and observed similar results indicating that total levels of rRNA molecules seem to be reduced in response to Mn. Next, we investigated the ribosome profiles of the cells in response to Mn treatment (Fig 5-3B). We observed a reduction in the pool of polysomes in response to treatment with 3 mM Mn for 1 hr, in addition to an increase in the pool of 80S ribosomes. Reduction in polysomes is interpreted as a decrease in the number of ribosomes that are active and engaged in synthesizing proteins. An increase in 80S monosomes is generally regarded as stalled initiation of translation. Treatment of the cells with Mn for 24 hrs, resulted in additional reduction in polysomes in comparison to control conditions. Lastly, using an expression vector we observed investigated the expression of β -galactosidase, used as a reporter, in

response to Mn. In a dose- dependent manner, we observed that the presence of Mn^{2+} reduced the expression of β -galactosidase, (Fig 5-3C). Importantly, this trend differs significantly when other divalent ions such as Ca^{2+} , Mg^{2+} and Zn^{2+} are used suggesting that the decreased rate of translation is unique to Mn^{2+} heavy metal stress and not a general byproduct (Appendix F, Fig F-2). Altogether, these follow up investigations connect Mn toxicity to the process of protein biosynthesis, which were identified as an enriched cellular process in our GDA analysis.

5.4.2 Manganese-induced disturbance of processes that converge to protein biosynthesis in yeast, which mimics molecular pathways associated with neurodegeneration

It has been postulated that the ER has various domains and membrane contact sites that are required for multiple cellular processes including synthesis of proteins and lipids, calcium regulation, and the exchange of macromolecules (English and Voeltz, 2013). In this study, through various approaches such as GDA (Fig 5-1); PPI (Fig 5-2), GO ontology (Table 5-1 and Appendix E, Table E-2 – table E-5) as well as analysis of total rRNA, Polysome profile and β -galactosidase assay (Fig 5-3), we suggested that the manganese induces perturbation of protein biosynthesis and associated pathways.

Figure 5-3: Influence of Mn on protein biosynthesis. A) Total RNA (1 μ g) is ran on a 1.2% agarose gel. rRNA levels are reduced in response to Mn (1.5 and 3 mM), for 0.75, 3 and 24 hours. B) Polysome profile analysis suggests that the number of active polysomes are reduced in response to Mn 3 mM, for 1 and 24 hours. C) The relative expression of β -galactosidase is reduced in response to the presence on increasing concentrations of Mn. Bars represent the mean value of at least 3 independent experiments and error bars represent (mean $\pm$ SEM). Differences were stipulated by one-way ANOVA and in specific cases by t-test analysis between two treatments. Letters indicate statistically significant differences among treatments ($p < 0.05$).

Taking into account these results, we selected several genes linked to the identified processes that converge on protein biosynthesis and then analyzed their relative transcription by qPCR (Fig 4). Indeed, the presence of manganese induced alteration these genes. For example; we observed down-regulation of key translation initiation factor eIF4A (*TIF1*) and up-regulation of the essential translation elongation factor eIF-5A (*HYP2*). Additionally, several other genes associated with translation and/or ribosome biogenesis had significantly altered levels of transcription including the down-regulation

of *NSR1*, *NPO1*, *RPL14A* and *RPL14B* and up-regulation of the gene *RPS15*. However; protein biosynthesis is a complex process that involve other pathways. For instance, we observed down-expression of the genes *OST2*, *UFD1*, *UFD2* and up-expression of the genes *OST6*, *STT3*, *DSK2* linked to protein processing in endoplasmic reticulum. Similar behavior was verified for N-glycan biosynthesis through down-regulation of *OST2* and up-regulation of *OST6* and *STT3*. Additionally; we observed down-expression of the gene *ARG3* involved in other important processes for protein synthesis such as amino acid biosynthesis, including arginine and proline metabolism (APM) and alanine, aspartate and glutamate metabolism (AAGM) as well as up-expression of the gene *URA2* associated with AAGM.

Figure 5-4: Relative gene-transcription of preselected hits from yeast stressed with manganese, after analysis of protein-protein interaction network (Fig 5-2), β -galactosidase assay and polysome profile, which are an evidence of perturbation of protein biosynthesis and other associated pathways. Analysis was performed by qPCR. Bars represent the mean value of at least 3 independent experiments and error bars represent (mean $\pm$ sem). Significant difference between Mn treatment and the control were verified by ANOVA one-way, followed of Bonferroni post-test and alternative t-test (*, $p < 0.05$).

Also, of interest, genes such as *CDC20*, *UFD2* and *UBC11*, which are related with ubiquitin proteolysis were significantly disrupted. Finally; protein synthesis underpins much of cell growth and their multiplication⁴³. Coincidentally; we observed a direct relationship among dysregulation of protein biosynthesis and significant impairment of genes such as *CDC20*, *CLB2* and *MCM2*, which are associated with MAPK signaling pathways, cell cycle and DNA replication respectively.

5.4.3 Mn-induced molecular impairment in yeast mimics pathways associated with neurodegeneration

We identified alterations of various pathways that lead to impairment of protein biosynthesis, which is a conflicting topic in neurodegeneration research. In some reports have viewed this as a therapeutic target, while others suggest that it provokes the onset of certain neurodegenerative disorders (Dasuri *et al.*, 2013). Due to the conservation of the key cellular processes and genes, yeast has been used as a model organism to study human neurodegenerative diseases (Braun *et al.*, 2010). In this sense, we conducted an additional analysis of the pathways affected by Mn using both the String database and the Comparative Toxicogenomics Database – CTD (Negga *et al.*, 2011), which permits the development of novel hypotheses about the relationships between chemicals and diseases (King *et al.*, 2012). The results are shown in Figure 5-5.

al., 2011). Additionally, it is well known that *CDC20* is linked to cell cycle and MAPK signaling pathways and potentially involved in the development of neurodisorders such as AD, ALS, HD and PD (Negga *et al.*, 2011). At the same time, it is known as well that the gene *UFD1* is associated with protein processing in endoplasmic reticulum and potentially linked to the evolution of AD, ALS and PD (Negga *et al.*, 2011). Altogether; our finding and inferences suggested that developmental impairment induced by manganese, according to cell cycle disruption, is mainly influenced by collective perturbation of pathways that converge to disturbance of protein biosynthesis. Since; we verified decreased of total RNA, polysome and beta-galactosidase activity as well as alteration in the expression of genes directly associated with translation (*HPY2* and *TIF1*) and ribosome biogenesis (*RPS15*, *RPL14A*, *RPL14B*, *NSR1* and *NOPI*). These facts suggested a plausible hypothesis for Mn-induced neurotoxicity and neurodegeneration (Fig 5-5B). Further analysis in other superior animal models are needed to elucidate this possibility.

5.5 Discussion

The role of Mn in toxicity; especially in neurotoxicity and neurodegeneration is unclear with several proposed hypotheses (Tarale *et al.*, 2016). In contrast; the Comparative Toxicogenomic Database, introduces Mn as an essential trace element, with possible connections to approximately 570 biological processes and/or pathways (Davis *et al.*, 2016). In this work, using a functional genomics and systems biology approach we observe a connection for Mn to cellular processes such as cell cycle, cell signaling pathway and protein metabolism. Agreeably, previous studies have suggested the

possibility that Mn may disturb cellular development processes (Kumar *et al.*, 2014; Tuormaa, 1996) as well as the flow of genetic information that could influence protein synthesis (Supek *et al.*, 1977; Dambach *et al.*, 2015), including ER stress (Wang *et al.*, 2015; Rudgalvyte *et al.*, 2016)

Our global chemical-genetic sensitivity screen, followed of GO term enrichment analysis suggested that Mn disturbed metabolic pathways, which includes anabolism or production of new compounds, usually through the processes that require energy. In line with this, we provided evidence to suggest alteration in the biosynthesis of amino acids (BAA) due to down-expression of the gene *ARG3*, involved in the biosynthesis of arginine from ornithine carbamoyl-transferase (Jauniaux *et al.*, 1978). Previous works have suggested that ornithine deficiency causes hyperammonemia and neurotoxicity, in humans (Mao *et al.*, 2011). At the same time; up-expression of the gene *URA2* is involved in the metabolism of pyrimidine, alanine, aspartate and glutamate (Denis-Duphil, 1989; Lue and Kaplan, 1969). Specially, alteration of arginine and proline metabolism has been associated with development of Amyotrophic Lateral Sclerosis (Patin *et al.*, 2017). The impairment of BAA can directly disrupt translation efficiency (Hu *et al.*, 2015), a process that is energetically very costly (Warner, 1999; Thomson, 2013). Furthermore, these events appear to be associated with inactivation of MAPK pathways that can lead to translation repression as well (Denis-Duphil, 1989; Warner, 1999) , antiapoptotic activities (Darling *et al.*, 2014) and/or cell cycle arrest (Warner, 1999; Darling *et al.*, 2014; Polymenis and Aramayo, 2015). In fact; we identified and inferred significant impairment of genes involved in cell cycle and MAPK pathways (Table 5-2 and Fig 5-2) such as *CDC20*, *CLB2* and *MCM2*, which was verified by q-PCR too. Cell cycle

disruption, which have been associated with AD, PD and ALS (Liu and Ander, 2012). At the same time; aberration from strict control of MAPK signaling pathway has been implicated in the development of different human diseases including AD, PD and ALS (Kim and Choi, 2010; Darling *et al.*, 2014).

According to the results discussed above, Mn-induced toxicity in yeast appears to be associated with essential pathways linked to protein metabolism. In the current study we infer that Mn may induce ER stress (Fig 5-2), which was demonstrated through qPCR analysis showing up-regulation of *DSK2*, *STT3* and *OST6* and down-regulation of *OST2* (Fig 5-4). This is in agreement with previous studies in vitro and in yeast that suggested that ATPase activity of the ER proteins *SPF1* is compromised under exposure to Mn^{2+} resulting in severe ER stress (Cohen *et al.*, 2013). Other works have suggested that Mn-induced ER stress can be mediated via iron depletion, increased phosphorylation of the eukaryotic translation initiation factor 2 α (phospho-eIF2 α) (Seo and Wessling-Resnick, 2013), activation of *PERK* and *IRE1* signaling pathways (Xu *et al.*, 2013; 2014) and ER tumefaction (Wang *et al.*, 2015). Recent studies using RNA-Seq approach revealed that in *Caenorhabditis elegans*, Mn induced up and downregulation of ER-related protein families (*FKB* and *ABU*) implicated in ER stress (Rudgalvyte *et al.*, 2016). ER stress can trigger a signaling reaction known as the unfolded protein response (*UPR*), which induces adaptive programs that improve protein folding. In certain neurodegenerative diseases such as AD, ALS, HD and PD, when the cell damage is irreversible, *UPR* can also activate apoptosis (Hetz and Saxena, 2017; Fruhmann *et al.*, 2017).

Moreover, we found that Mn could potentially influence glycosylation as well

because dysregulation of the genes *OST2*, *OST6* and *STT3*, which are linked to the ER. This is in agreement with previous observations, by others (Park *et al.*, 2015; Potelle *et al.*, 2016). Various studies suggested that aminoglycoside antibiotics that induce errors in post-translational modification like glycosylation and the misfolding of proteins can lead to destabilized membranes and chronic stress (Drummond and Wilke, 2009). Other studies suggested that alterations of *SLC39A8* links Mn deficiency to inherited glycosylation disorders, specifically impairment of Mn-dependent enzymes activity, most notably the Golgi enzyme β -1,4-galactosyltransferase, which is essential for biosynthesis of the carbohydrates in glycoproteins (Park *et al.*, 2015). Moreover, Golgi glycosylation defects may also be as a result of *Gdt1p/TMEM165* deficiency that stem from Golgi Mn homeostasis defects (Potelle *et al.*, 2016). Collectively, these evidences suggest that ER stress, in yeast treated with Mn, can be associated with impairment of N-glycan biosynthesis (Ruddock and Molinari, 2006), which consequently may lead to arrest the protein biosynthesis.

Additionally, ER stress can be exacerbated by impairment of endosome-to-Golgi retrograde trafficking (Johannes and Popoff, 2008). Since the retromer complex, constituted by vacuolar protein sorting (VPS), is essential to the bidirectional transport between the trans-Golgi network (TGN) and endosomes, it is one of the key vesicular trafficking pathways in the cell (Progida and Bakke, 2016), including transport of protein to endoplasmic reticulum (Bonifacino and Rojas, 2006; Johannes and Popoff, 2008; Tsvetanova, 2013). Indeed; we identified significant disruption of *VPS5* (Fig 5-1B), and the STRING database added other protein/genes with similar function such as *Vps35*, *Vps29*, *Vps17* and *PEP8* (Fig 5-2). Vesicle transport have been considered to play an

important role in yeast and mammalian models of Amyotrophic Lateral Sclerosis as well (Van Damme *et al.*, 2017). Furthermore; other studies have verified Mn down-regulated the expression of *SNAP-25* and up-regulate the expression of *VAMP-2*, which interacted with Synaptophysin (Wang *et al.*, 2017). Using the FM1-43 dye (N-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino) Styryl) Pyridinium Dibromide) that is an excellent reagent both for identifying actively firing neurons and for investigating the mechanisms of activity-dependent vesicle cycling (Lee and Pappone, 1999), various authors verified that FM1-43-labeled synaptic vesicles treated with Mn resulted in an initial increase followed by a decrease in the number of vesicles (Wang *et al.*, 2017). Other studies linked Mn neurotoxicity to disruption of genes with transport functions including *SLC30A10*, *ATP13A2* and *ZnT10* (Leyva-Illades *et al.*, 2014; Nishit *et al.*, 2016). At the same time; it is well known that Golgi dysfunction can lead to rapid repression of rRNA and ribosomal proteins (Warner, 1999), affecting the protein biosynthesis. Indeed; we observed translation arrest in yeast was notably increased after 24 hours exposure to Mn (decreasing of β -galactosidase activity), suggesting that long or chronic exposure of Mn^{2+} appears more effective in yeast than short or acute exposure; this is similar to observations made by others (Hernández *et al.*, 2011; 2015). We conducted the same analyses using other divalent cations such as Ca^{2+} , Mg^{2+} and Zn^{2+} at equivalent or higher concentrations to determine if this was a general effect of 2+ light/heavy metal stress and did not observe decreased rates of expression like the dose-dependent response seen to Mn^{2+} in figure 5-3C. In addition, our ribosome profile analysis revealed a reduction in heavy polysomes fractions in response to Mn suggesting that Mn reduces efficiency of translation (Fig 5-3B). This may be in agreement with a

recent study in Human SH-SY5Y cells that identified Mn-induced ER stress associated with increased phosphorylation of translation initiation factor *eIF2 α* (Seo and Wessling-Resnick, 2013). Similar profiles have been reported when anti-translation drugs including pactamycin and harringtonine (Al-Jubran *et al.*, 2013).

Furthermore, we verified direct impairment of protein biosynthesis, including disruption of the translation factor *TIF1* and of ribosome biogenesis due to down-regulation of the genes *NOPI*, *NSR1*, *RPL14A* and *RPL14B*; although this can be partially composed through up-regulation of the genes *HYP2* and *RPS15*. A review at the CTDc (Davis *et al.*, 2016) revealed that *RPS15* is a marker of Disease Progression, including memory impairment in transgenic mice of Alzheimer's disease treated with copper (Yu *et al.*, 2015) as well as RPL14 is a marker of Parkinson Disease, which have been observed in case of residential exposure to maneb and paraquat (Costello *et al.*, 2009). It is very interesting because, while the paraquat is a 1,1'-dimetil-4,4'-bipiridina-dicloreto, the maneb is a polymeric complex of manganese with the ethylene bis (dithiocarbamate) anionic ligand. Unpublished studies of our group have identified the maneb-induced impairment of protein biosynthesis in cerebellar granule neurons.

5.6 Final remarks

Literature in the field has consolidated robust hypotheses about manganese induced-neurotoxicity and neurodegeneration that include mitochondrial dysfunction, energy impairment, oxidative stress, disruption of neurotransmitters, ER stress, neuroinflammation, DNA damage and epigenetic alterations, apoptosis, autophagy, among others (Peres *et al.*, 2016; Pfalzer and Bowman, 2017; Tarale *et al.*, 2017; Bevan

et al., 2017). However, occasionally, the accuracy these hypotheses is challenged in different models. All processes cited above either occur after the process of protein biosynthesis is completed and/or are directly linked to it. In the current study we identify protein biosynthesis as a key target of Mn-induced toxicity in yeast among others. Defects in protein biosynthesis has been documented in different neurodegenerative disorders in humans. Since Mn-induced toxicity has been linked to human neurodegenerative disorders, the data presented in the current study may provide a connection between Mn-induced toxicity and human neurodegenerative disorders through the process of protein biosynthesis. Altogether, our findings provide strong evidence that Mn-toxicity can occur in multiple and simultaneous levels, which appear to be associated with disruption of orchestrated essential pathways including metabolism and protein biosynthesis. In this way the current study adds to our current understanding of the mode of toxicity of this metal. Additional experiments with mammalian models must be conducted to validate these findings.

6 Chapter 6: General conclusion

6.1 Final conclusion and discussion

Investigation and understanding the molecular mechanisms of cellular responses and detoxification processes that overcome the toxicity of these compounds in living organisms is very important. Many studies in the past addressed this task and several genes are identified to play central role in the cellular detoxification process (Rai and Cooper 2005; Violante *et al.*, 2010; Jaishankar *et al.*, 2014; Moteshareie *et al.*, 2018). For example, in *S. cerevisiae* Ureidosuccinate Transport 2 (*URE2*) encodes for a protein that functions as a glutathione peroxidase activity and is homologous to mammalian glutathione S-transferases. The resistance of *S. cerevisiae* to a wide range of heavy ions is synchronized by glutathione S-transferase product. Gene deletion of *URE2* exhibits hyper-sensitivity to various metals and metalloids including Cd and As (Todorova *et al.* 2010; Moteshareie *et al.*, 2018). The expression of such genes can be regulated at the transcriptional and/or translational levels in response to the presence of heavy metals. Such effect, in the presence of toxic metals, is facilitated by numerous factors that can regulate the expression of genes that are directly involved in the cellular detoxifications.

Chemical genomics is a relatively novel approach that in recent years has received a considerable attention due to its ease of use and ability to generate a significant amount of targeted data in a relatively short period of time (Burnside *et al.*, 2014). Also known as chemogenomics, it is used to study the mode of activity of various compounds as well as investigating novel gene and protein functions. The field of chemical genomics is predicted to play a critical role in studying compound toxicity, development of novel

noninvasive drug discovery and biological research. For example, using yeast deletion set array, Márquez *et al.*, in 2018 assessed the toxic effects of two commonly used engineered nanoparticles, silver nanoparticles (AgNPs) and zinc oxide nanoparticles (ZnONPs) on yeast cells. A library of approximately 4500 non-essential yeast, *S. cerevisiae*, mutant strains was utilized to infer the genes, whose absence makes *S. cerevisiae* more sensitive to the cytotoxic effects of both compounds (Márquez *et al.*, 2018). Yeast chemical screening process is a time-effective and relatively low-cost method that allows for simultaneous examination of an array of ~4500 individual mutants strains (Florian *et al.*, 2007). Recently, the high-throughput screening techniques have been developed to perform up to 12 thousand assays at once in nanowells containing smaller reaction volumes; in this way the efficiency of the experiment is increased, and the cost of materials is also lowered (Stockwell, 2000; Rubilar-Hernández *et al.*, 2014).

In the current study, while screening for yeast gene deletion mutants (~4500) against heavy metals and metalloids, we identified four deletion mutant strains for *ITT1*, *RPS1A*, *UGA4* and *YBR062C* that showed increased sensitivity to all three tested heavy metals (As, Ni and Cd). Remarkably, no connection to heavy metal toxicity has been previously reported for either of these genes, making them interesting target for further investigations. The mechanisms by which they inferred resistance was the focus of this thesis.

For my studies, yeast was chosen as the model organism. the baker's yeast, *S. cerevisiae*, has demonstrated to be a reliable eukaryotic model organism in countless studies. It has been extensively used to systematically investigate numerous cellular

processes in living organisms. Over the past decades, several libraries of gene deletion arrays for both yeast mating types (*MATa* and *MAT α*) have been developed including haploid deletions, heterozygous and homozygous diploid deletions and GFP infused library. In addition, availability of different phenotypic experiments for yeast, made this model organism, an ideal organism for functional genomics investigations. These experiments link phenotypes associated with the loss of functions to several gene deletions in systematic genome-wide investigations. One of the most substantial problems that challenges scientists in this field remains the significant rates of false positive and negative that can be directly linked to the size of the produced data. False positives represent the data that is produced as positive hits but were not true positives. In parallel, false negative results are the data that was not produced as positive hits in a screen method but were actually true positives. The rate of false positives and false negatives can vary between ~10 - 50 % for many large-scale experiments. For example, the normal rate of false positives for general Yeast Two Hybrid (Y2H) data is thought to be as high as ~45-50% (Delneri *et al.*, 2001; Phelps *et al.*, 2002; Oliver *et al.*, 2002; Urbanczyk-Wochniak *et al.*, 2003; Venancio *et al.*, 2010; Jessulat *et al.*, 2011). A large number of positive hits were observed from our high-throughput heavy metal screening methods. Many of these hits may not be reliable. A logical way to increase the reliability of the data is to maintain consistent statistics of the effectiveness of the used technique. This may be achieved by running preliminary experimental analysis in which a reasonable number of positive and negative controls are included. In this way, the sensitivity and specificity of the used technique(s) can be calculated and screening conditions can be adjusted accordingly. Using the growing number of freely available online data bases from similar

large-scale experiments is a common practice in yeast functional genomics to estimate statistical analysis. Certainly, optimal experimental conditions can reduce the rate of false negative results however regardless of the efforts dedicated to minimizing the rate of false positive data by improving experimental conditions, it is always imperative to support the produced results collected from high-throughput screening investigations by using more directed follow-up smaller-scale experiments. In the current study I used β -galactosidase blue and white lift assay for the initial large-scale screening which was followed up by small-scale ONPG-based quantitative β -galactosidase liquid assay. After large-scale screens, potential positive hits can be subjected to smaller scale more directed and reliable experiments and only the reconfirmed positive hits can be subjected to more detailed investigations which can often save time and costs associated with these kinds of examinations.

To this end, our more directed investigations into the role of our four genes that were originally identified in our large-scale heavy metal screens, demonstrated an indirect role through expression of a detoxifying gene known as *URE2*. All four knockout strains exhibited similar drug sensitivity phenotypes to the knockout strain for *URE2*. Our extensive gene expression analysis indicated that *ITT1*, *RPS1A*, *UGA4* and *YBR062C* significantly affect the translation rate of *URE2*, specifically at IRES-mediated translation of *URE2*/IRES element. These novel gene functions help to provide a more comprehensive understanding of the mechanisms that contribute to effective heavy-metal detoxification strategies at the cellular level. We complimented our investigations by analyzing the genetic interaction networks formed by these candidates through SGA and PSA. Genes that are functionally related often partake in GIs (also known as epistatic

interactions) (Rienger *et al.*, 2012; Szendro *et al.*, 2013). Our conditional genetic interaction analysis has indicated the involvement of all four genes mainly in regulation of translation in the presence of the metals, connecting their activity to the process of translation. PSA results constitute a more direct form of GI and can infer close functional relationships between interacting genes. Our PSA results provided further evidence connecting our four candidate genes to IRES-mediated translation.

Protein synthesis is one of the most fundamental cellular processes, and it is highly regulated within a living cell. In addition to canonical cap-dependent translation, certain mRNAs can be translated independent of 7-methylguanosine cap and certain associated factors via internal structures known as IRES. This kind of cellular translation initiation is normally utilized under stress conditions where the canonical cap-dependent translation is compromised, mainly as a survival mechanism. Although over the past few decades of investigations, a wealth of information has been obtained on the process of protein synthesis, there is a gap of knowledge in understanding the comprehensive details of mechanisms that govern the translation process, specifically internal initiation of translation as well as the cross-communications between translational components and other cellular factors and processes (Pestova *et al.*, 2007; Babu *et al.*, 2011; Zhao *et al.*, 2017; Samanfar *et al.*, 2018). A growing list of factors that have an influence on the process of protein synthesis further supports the existence of additional novel factors in this process. In the recent years, an effective large-scale plasmid-based method has been developed to investigate specific gene deletion effects on translation process (e.g Samanfar *et al.*, 2014). This method utilizes a modified version of the SGA technique to systematically transfer plasmids carrying four hairpin loops followed by an IRES forming

region fused to a *β-galactosidase* expression cassette along with another plasmid with no hairpin loops nor IRES region (as a control) into a yeast non-essential gene deletion collection (~ 63,000 transformations). This was then followed by X-gal colony lift assay. In this context, we were interested in novel genes that can nonspecifically influence the process of eukaryotic IRES elements hence we would be able to assign them a novel function in the protein synthesis pathway. It is noteworthy that our large-scale screening is by no means exhaustive and there still may exist additional factors that nonspecifically affect IRES-mediated translation in yeast. Depending on the intensity of their influence, our screen may miss factors that have little effect under normal condition. Other factors may simply affect IRES-mediated translation under specific conditions that were not tested in our screen. For example, certain IRES-mediated translation can be regulated under heat shock, requiring a different set of factors.

In the current work, we have successfully sub-cloned 10 yeast IRES forming regions along with two IRES regions found in human p53 gene. Subsequently, they were transformed into yeast *MATα* for further confirmation of their translation activity. Of 12 transformed strains five were shown to exhibit notable signal from *β-galactosidase* activity under translational control of the inserted five IRES regions. Of these, four IRESs along with the original *URE2*-IRES were selected for follow up large-scale screen. We sought to identify novel genes encoding regulatory factors known as IRES *trans*-acting factors (ITAFs). We identified a total of 142 novel genes with specific or nonspecific potential ITAF activities. A total of 23 candidate genes with a potential role in non-specific regulation of IRES-mediated translation for two or more IRES elements as were identified. Of these 23 candidate genes, *HAP3*, *COX5A*, *CDA2*, *THP2*, and *NCE102* were

selected for further investigations. Unlike viral IRESs that have been classified to four types, eukaryotic IRESs are so diverse that makes them impractical to cluster into different groups (Komar, and Hatzoglou, 2011). Each cellular IRES holds a unique structure and requirements for specific ITAFs. The fact that we identified a large list of potential specific ITAFs, suggests a highly regulatory process for each individual IRES element. Our preliminary attempts to identify a unifying potential pathway that majority of our ITAF candidates can be enriched for failed. In addition, of a list of 142 potential ITAFs, we only identified 23 candidates to be common between at least three IRES elements, five between five IRES elements and only one between eight IRES elements. The Lack of nonspecific ITAFs suggests the complexity of cellular IRESs and adds to the diversity of the factors involved in this process. This finding in yeast may also suggest the same complex scenario for human IRESs.

The YKO is a general and unbiased collection of non-essential deletion mutants hence in theory, with the exception of essential genes, there is no bias towards genes of specific functions. Using an array containing deletion mutants of genes previously discovered or postulated to have ITAF function, as well as their interacting genes/proteins, we would have generated a biased array that could potentially identify a higher proportion of positive hits. However, the scope of that approach would have been limited. In order to support our positive hits and reduce false positive rates, we subjected all the selected candidates to ONPG-based small-scale quantitative β -galactosidase assay. The large-scale X-gal lift assay is not a quantitative method, and more error prone whereas the ONPG-based β -galactosidase assay is a low-throughput quantitative assay method with less susceptibility to error. Based on our quantitative β -galactosidase assay

for 142 identified genes we estimated around 15% false positives for our large-scale screening. Estimation of the false negatives however is significantly more challenging as it is often difficult to estimate the complete number of true positives.

Overall, our investigations reaffirm the usage of large-scale analysis in the field of functional genomics and translation to identify novel components. Future follow up experiments are required to unequivocally establish the role of these candidate genes as nonspecific ITAFs in a eukaryotic context. Western blot experiments should be carried out to analyze the involvement of identified candidate genes on the relative endogenous levels of IRES-mediated translation of *FLO8*, *GPR1*, *YAP1*, *NCE102* and *URE2* with respect to wild type. Potential results from this experiment can verify the regulatory functions of these genes in IRES-mediated translation. These results can then be paired with polysome profiling in the absence of the identified potential ITAFs to uncover more about the translational activity of these factors.

In parallel, reintroduction of the candidate genes into the knockout strains used for β -galactosidase activity assays, in case of compensation in the reduced β -galactosidase activity, would also provide a compelling support for the observed phenotype not to be an artefact of a potential secondary mutation. Given the high rate of false positives and negatives which techniques such as the colony lift assay in large-scale screen yields, it may be beneficial to investigate genes from overrepresented families in the lists of positive hits obtained from the large-scale screening. This should include genes found through any of the three colony lift replicates for each IRES element. In this way, it may be possible to find potential candidates which did not necessarily exhibit a promising

phenotype throughout the colony lifts. The presence of several genes from the same family may indicate relation between the process represented by said family and that of IRES-mediated translation. Although no gene family stands out from our screen's results, this may no longer be the case if the screens were to be repeated.

Studying novel translation-related genes in more complex eukaryotic organisms such as human, based on their yeast homology provides a suitable opportunity to better understand and investigate the details of this important process in humans. Investigation into the interaction networks that the discovered novel ITAF genes form, can also be used to identify related genes and establish whether the identified interacting partners also play translational roles. Finally, the interactions between our four target IRES elements and the potential ITAFs encoded by the candidate genes we identified should be studied in the context of biomolecular interaction studies. Other techniques including surface plasmon resonance and RNA-based pull-down experiments can be performed in the future to characterize the candidates' regulatory role. Unconfirmed newly constructed plasmids for the IRES activity can also be tested under additional IRES-inducing treatments such as heat shock (Kim and Jang, 2002; Samanfar *et al.*, 2017). In case any of other IRESs are confirmed to be functional under the new experimental conditions, we can use them for new large-scale screenings. In addition, we can also screen for the genes that their absence can upregulate IRES-mediated translation. Over all, we believe that these results may lead to further work aiming to elucidate distinct IRES mechanisms shared between different cellular IRESs. Translation is one of the most extensively studied cellular processes. The fact that we identified a number of novel factors that can affect such an extensively studied pathway suggests the possibility of the existence of other novel

factors that may affect other cellular processes. This underlines the need for future functional genomics investigations.

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7 Appendices

7.1 Appendix A

Supplementary data

Figure A1: Normalized CFU counts for different yeast strains after exposure to acetic acid (180 mM), heat shock (37°C), and MMS (0.05% v/v). CFU counts after 3 days exposure to the experimental conditions are normalized to control condition counts. Sensitivity of *itt1Δ*, *rps1aΔ* are compared to WT phenotype in the presence of (A) acetic acid (180mM), (B) heat shock (37°C) and (C) MMS (0.05% v/v). For each experimental condition, a corresponding positive control strain is included. Each experiment was repeated at least three times. Error bars represent standard deviations. * Indicates statistically significant differences ($p < 0.005$).

Figure A-2: Spot test sensitivity analysis for different yeast strains. Cells were grown to saturation, serially diluted, and spotted on solid media containing As(III) (1 mM), Cd (0.1 mM), Ni (8 mM) or no drug. Plates were incubated at 30°C for 3 days.

Figure A-3: Spot test sensitivity analysis of selected GIs. Cells were grown to saturation, serially diluted, and spotted on solid media with or without As(III). (A) and (B) *EDC1* and *DOM34* show conditional GI with *ITT1* and *RPS1A* in the presence of As(III) (0.7 mM) representing conditional negative genetic interactions. *DAN1* is used as a negative control. (C) and (D) Overexpression of *ITT1* and *RPS1A* compensated the sensitivity of gene deletion strains for *GIS2* and *DOM34*, respectively, in the presence of As(III) (1.2 mM) confirming a phenotypic suppression GI for *ITT1* with *GIS2* and *RPS1A* with *DOM34*. *DAN1* was used as a negative control.

7.2 Appendix B

Sequence-specific primers used for IRES element amplification

Table B-1 Restriction site in **blue** is for *Eco*R1; restriction site in **red** is for *Sac*II

IRES gene name	Amplicon length (bp)	Primer direction	Primer sequence (5' → 3')	Primer length (bp)	Melting temperature (°C)
<i>BOII</i>	204	Forward	TAC CCGCGG GTCTGTCTAAAGAAACAATTGT	30	63.0
		Reverse	TAGAATTCATCACCTGGTTTCATATTGAGCTC	32	59.5
<i>FLO8</i>	208	Forward	TAC CCGCGG AGTTGACGTTAGTAAGTCA	27	62.7
		Reverse	GCGAATTC CA CTATTTCGTTGCCATTGC	27	63.0
<i>SPT15</i>	304	Forward	TAC CCGCGG AGGGACGTGAAAATTACA	26	63.9
		Reverse	GCGAATTCGTAGTTGCTGGTTTGT	25	59.9
<i>GIC1</i>	197	Forward	TAC CCGCGG CTTTTTTTAAGAAAGACTT	28	58.8
		Reverse	TAGAATTCATGGATTCCAAGTAAGGCA	28	56.7
<i>GPRI</i>	180	Forward	TAC CCGCGG AAAAAACGACAAACAAGTGA	29	62.6
		Reverse	TAGAATTC CA AACCCAGCAGCTGGTTAA	28	63.5
<i>TIF4631</i>	623	Forward	TAC CCGCGG TCATTTTTTTGAAGATTACA	27	58.2
		Reverse	TAGAATTCGTAGTTGGTGTAGCCACG	26	59.8
<i>HAP4</i>	397	Forward	TAC CCGCGG ACCTCTCTAAACCCAGTTT	28	66.8
		Reverse	GCGAATTCGGTTGGTATTTGGGGCGATC	28	66.4
<i>YAP1</i>	288	Forward	TAC CCGCGG CCGATTAAGCACAGTACCTT	28	67.3
		Reverse	GCGAATTCACGTGTACCAGTACGTCT	27	64.0
<i>MSN1</i>	210	Forward	TAC CCGCGG TAAAGTGTTCTGATTCATTA	28	59.2
		Reverse	TAGAATTC TA ACGCGTGAAATATACCCTC	29	57.6
<i>YMR181C</i>	210	Forward	TAC CCGCGG GGATTGTTTATAAGGATTTT	28	59.8
		Reverse	TAGAATTC TAC CGAGGGAGAAGAATAGA	28	57.5
<i>NCE102</i>	212	Forward	TAC CCGCGG AACATTCTTTAAAAAACATCTT	31	58.5
		Reverse	TAGAATTCAGTAGTTTACTCTGGAGCTGT	30	59.6
p53 ¹ (FL)	501	Forward	G CCCGCGG AACAATGAATAAGATAGTAGA	28	61.9
		Reverse	TAGAATTC CA ACCCCTGCGAGGCTCCT	27	67.5
p53 ² (ΔN)	251	Forward	TAC CCGCGG CTAGAGCCACCGTCCAGGGA	29	73.7
		Reverse	TAGAATTC TG CTTGGGACGGCAAGGG	26	65.2

7.3 Appendix C

DNA sequencing results for p281-4-IRES plasmid series via Sanger method

Sequences were determined through alignment performed using NCBI Basic Local Alignment Search Tool (BLAST®).

Sample 1: p281-4-*FLO8*

Forward amplification:

5'{GTTGATTACTACATATCACGATTACGTTTATAGACATAAATAAAGAGGAAA
CGCATTCGGTAGAATGAGTTATAAAGTGAATAGTTCGTATCCAGATTCAA
TTCCTCCACGGAACAACCGTACATGGCAAGCCAGTATAACAAGATTTGCA
GAGTAATATTGCAATGGCAACGAATAGTGGAATTCCCGGGGATCCCGTCGTT
TTACAACGTCGTGACTGGGAAAACCTGGCGTTACCCAACTTAATCGCCTTGC
AGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGAT
CGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGTT
TCCGGCACCAAAAGCGGTGCCGGAATTTTGGCTGGCCCCCTCTTCCTGAGG
AAAAAAATGTTTTTTTTTTTTTCGGCTGGCCACGAACAGCCCCCCCCCCCCCT
TCTTT}3'

Reverse amplification:

5'{AAATAAATCATCTGGCAATTCTTGTTTATACTGGCTTGCCATGTACGGTTGT
TCCGTGGGAGGAATTGAATCTGGATACGAAAAATTCACTTTATAACTCATTCT
ACCACGGAATGCGTTTCCTCTTTATTTATGTCTATAAACGTCTTCGTGTTTATT
TTTTATAGCCTCAGTGACTTACTAACGTCAACTCCGCGGAGATCCTAGGATCC
TAGGATCCTAGGATCCTAGGATCTTAACAATTTTTTGTGATACTTTTATTACA
TTTGAATAAGAAGTAATACAAACCGAAAATGTTGAAAGTATTAGTTAAAGTG
GTTATGCAGTTTTTGCATTTATATCTGTTAATAGATCAAAAATCATCGCTTC
GCTGATTAATTACCCAGAAATAAGGCTAAAAAACTAATCGCATTATCATCCT
ATGGTTGTTAATTTGATTCGTTTCATTTGAAGGTTTGTGGGGCCAGGTTACTGC
CAATTTTTCCTCTTCATACCCCCCAAAGCTAGTATTGGAGAATCTTTATTGTTC
GCCGTCTTCCGGCGCGAGGCACACCTGCGTTTCCGGCGCGCCTTTTTTTCATA
CGAGGCCGCGCGCATCAGTCCCTCTTCCCACCCACGTCCCACTATAGCCCA}3'

Sample 2: p281-4-*HAP4*

Forward amplification:

5'{GGATCGGTAATGCTATCTACAGGTCCACTTTACACTTAATAATATAAAAAT
ACTACTATAAAGGAACCAGAAAAATAAAAAAGGGTCATTATTTATTTGAGCA
GATCATTATCAAACGCATAGGAAGAGAAAAAACACAGTTTTATTTTTTTTCCA
CACATATTTATTGGTCTCCTAGTACATCAAAGAGCATTTTAATGGGTTGCTGA

TTTGTTTTACCTACATTTTCTAGTACGAAAAAAAAACAAAAAGAATCATGA
CCGCAAAGACTTTTCTACTACAGGCCTCCGCTAGTCGCCCTCGTAGTAACCAT
TTTAAAAATGAGCATAATAATATTCCATTGGCGCCTGTACCGATCGCCCCAAA
TACCAACCGAATTCCCGGGGATCCCGTCGTTTTACAACGTCGTGACTGGGAAA
ACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGC
TGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCA
GCCTGAATGGCGAATGGCGCTTTGCCTGGTTTTCCGGCACCAGAAGCGGTGCC
GGAAAGCTGGCTGGAGTGCATCTTCCTGAGGCCGATACTGTCCTTCCTCCCC
TCAAATGGCAGATGCCCCGTTACAATGCGCCCATCCACACCCCGTGA}3' 52

Reverse amplification:

5'{GTGGCAGCGCATGGATATTATTATGCTCATTTTTTAAATGGTTACTACGAGG
GCGACTAGCGGAGGCCTGTAGTAGAAAAGTCTTTGCGGTCATGATTCTTTTTT
TGTTTTTTTTTCGTACTAGAAAATGTAGGTAAAACAAATCAGCAACCCATTAA
AATGCTCTTTGATGTACTAGGAGACCAATAAATATGTGTGGAAAAAAAAAATAA
AACTGTGTTTTTCTCTTCCTATGCGTTTGATAATGATCTGCTCAAATAAATAA
TGACCCTTTTTTATTTTTCTGGTTCCTTTATAGTAGTATTTTTATATTATTAAGT
GTAAAGTGGACCTGTAGATAGCATATACGATATAAAACTGGGGTTTAGAGAG
GTCCGCGGAGATCCTAGGATCCTAGGATCCTAGGATCCTAGGATCTTAACAAT
TTTTTGTTGTTACTTTTATTACATTTGAATAAGAAGTAATACAAACCGAAAAT
GTTGAAAGTATTAGTTAAAGTGGTTATGCAGTTTTTGCATTTATATATCTGTTA
ATAGATCCAAAATCATCGCTTCCCCTGATTAATTACCCTTTTTTATAAGGCTA
AAAACTAATCGCATTATCATCCTATGGTTGTTCAATTTGATTCCTTCATTTGAA
GGTTCGTGGGGGCAGATTACTGCTACTTTTTCCCCTTAA}3'

Sample 3: p281-4-YAP1

Forward amplification:

5'{AGCAATAGGATGGTGTTTAGCTTTTTTTCCTGAGCCCCTGGTTGACTTGTGC
ATGAACACGAGCCATTTTGTGTTTAAAGGGAAGTTTTTGCACCCAAAA
CGTTTAAAGAAGGAAAAGTTGTTTCTTAAACCATGAGTGTGTCTACCGCCAAG
AGGTCGCTGGATGTCGTTTCTCCGGGTTTCATTAGCGGAGTTTGAGGGTTCAA
ATCTCGTCACGATGAAATAGAAAATGAACATAGACGTACTGGTACACGTGGA
ATTCCCGGGGATCCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGT
TACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATA
GCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGG
CGAATGGCGCTTTGCCTGGTTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGG
CTGGAGTGCATCTTCCTGAGGCCATACTGTCGTCGTCCCCCTCAAACCTGGCA
GATGCACGGTTACGATGCGCCCATCTACACCAACGTGACCTATCTCATTACGG
TCAATCCGCCGTTTGTTCACGGAAAATCCCAACGGGTTCGTTTCCCTCCTCA
CAT}3'

Reverse amplification:

5' {GTGAAAATTTTCAATTTCTCGTGACGAGATTTGACCCTCAAACCTCCGCTAA
TGAACCCGGAGAAACGACATCCAGCGACCTCTTGGCGGTAGACACACTCATG
GTTTAAGAAACAACCTTTTCCTTCTTTAAACGTTTTTGGGTGGCAAAAACTTCC
CTTAAACAAACTAAAAATGGCTCGTGTTTCATGCACAAGTCAACCAGGGGGCTC
AGGAAAAAAGCTAAACACCAATCCTATATATAACGTAAGGTACTGTGCTTA
ATCGGCCGCGGAGATCCTAGGATCCTAGGATCCTAGGATCCTAGGATCTTAA
CAATTTTTTTGTTGATACTTTTATTACATTTGAATAAGAAGTAATACAAACCGA
AAATGTTGAAAGTATTAGTTAAAGTGGTTATGCAGTTTTTGCATTTATATATC
TGTTAATAGATCAAAAATCATCGCTTCGCTGATTAATTACCCAGAAATAAGG
CTAAAAAACTAATCGCATTATCATCCTATGGTTGTTAATTTGATTCGTTTCATTT
GAAGGTTTGTGGGGCCAGGTTACTGCCAATTTTTCTCTTCATAACCATAAAA
GCTAGTATTGTAGAATCTTTATTGTTTCGGAGCAGTGCGGCGCGAGGCACATCT
GCGTTTCATGAACGCGACCGGTGAATACGAGGACGCACGGAGGAGAAGTCTT
CTCTTCGGAGGGCTGTCACCCGCTTGGCGGGCTTCTAATCCGTACTTCAATAT
AACAAATGACCAATTAAGCGTACTACTTAAAGTTCCCAAGAATAAGGTTTTCT
TTATCGCTAAAA}3'

Sample 4: p281-4-MSNI

Forward amplification:

5' {TTGCCCCTTTGGCTTATAAGAAAGAACCAAATCAGAAAAGGAGATTATTTTC
AAGGTAGGCATCGAAATGGCAAGTAACCAGCACATAGGAGCTTCAAACCTAA
ATGAGAATGAGGCTATATTAACC...AACCGCGTTGCTGAGCTGGAAAGGCGTA
TGTCGATGTTTGAGGGTATATTTACGCGTTAGAATTCCCGGGGATCCCGTCG
TTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACCTAATCGCCTT
GCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCCGCACCG
ATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGG
TTTCCGGCACCAGAAGCGGTGCCGGAAGCTGGCTGGAGTGCGATCTTCCTG
AGGCCGATACTGTCGTCGTCCCCTCAAACCTGGCAGATGCACGGTTACGATGC
GCCCATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTTC
CCACGGAGAATCCGACGGGTTGTTACTCGCTCACATTTAATGTTGATGAAAAG
CTGGCTACAGGAAGGCCAGACGCGAATTATTTTTGATGGCGTTAACTCGGCGT
TTCATCTGTGGTGCAACGGGCGCTGGGTTCGGTTACGGCCAGGACAGTCGTTTT
GCCGTCTGAATTTGACTTGAGCGCATTTTTTACGCGCCGGAGAAAAACCGCCT
CGCGGTGATGGTGCTGCGCTGGACTGACGGCACTTATCGGGAAGATCTGGAT
ATGTGGCGGATAACGGCTTTTTTCGTGACCTTCTTCTTTGGC}3'

Reverse amplification:

5' {TAAACCCCCCTACGCCTTTCAGCTCAGCACGCGGTTGGTTAATATAGCCTC

ATTCTCATTTAGGTTTGAAGCTCCTATGTGCTGGTTACTTGCCATTTTCGATGCC
TACCTTGAAATAATCTCCTTTTCTGATTTGGTTTCTTTTCTTATAAGCAAGGGG
CTTTGTATAATGAATCAGAACACTTTACCGCGGAGATCCTAGGATCCTAGGAT
CCTAGGATCCTAGGATCTTAACAATTTTTTGTGATACTTTTATTACATTTGAA
TAAGAAGTAATACAAACCGAAAATGTTGAAAGTATTAATTAAAGTGGTTATG
CAGTTTCTGCATTTATATATCTGTTAATAGATCAAAAATCATCGCTTCCCTGAT
TAATTACCCCGAGACCCAACGCTAAAAAACTCGTTCCGATTATTTTCTATGGCT
GCTCTTTC}3'

Sample 5: p281-4-NCE102

Forward amplification:

5'{CATAAGAAATCGGTTAGAAAACCTTTCTTCTCAAGCATACCTAATAACAAT
ATAATCCCATAATGCTAGCCCTAGCTGATAACATTCTACGTATAATAAATTC
CTATTTTTGGTTATTTCCATCGGTTTAATCAGTTCGTTGTTAAACACCCAACAT
AGGCACAGCTCCAGAGTAACTACTCCCAAAACGTTTAAAGAAGGAAAAGTT
GTTTCTTAAACCATGAGTGTGTCTACCGCCAAGAGGTCGCTGGATGTCGTTTC
TCCGGGTTTATTAGCGGAGTTTGAGGGTTCAAATCTCGTCACGATGAAATAG
AAAATGAACATAGACGTACTGGTACACGTGGAATTCCCGGGGATCCCGTCGT
TTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACCTTAATCGCCTTG
CAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGA
TCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGT
TTCCGGCACCAGAAGCGGTGCCGAAAGCTGGCTGGAGTGCGATCTTCCTGA
GGCCCGATACTGTCGTCGTCCCCTCAAACCTGGCAGATGCACGGTTACGATGCG
CCCATCTACCAACGTGACCCTATCCCATTACGGTCAATCCGCC}3'

Reverse amplification:

5'{TGGGGCCTTATGTTTGGGTGTTTACACGAACTGATTAAACCGATGGAAATA
ACCAAAAATAGGAAATTTATTATACGTAGAATGTTATCAGCTAGGGCTAGCA
TTATGGGATTATATTGTTATTAGGTATGCTTTGAGAAGAAAAGTTTTTTAAC
CGATTTTTCTTATTGAAGATGTTTTTTAAAGAAATGTTCCGCGGAGATCCTAG
GATCCTAGGATCCTAGGATCCTAGGATCTTAACAATTTTTTGTGATACTTTTA
TTACATTTGAATAAGAAGTAATACAAACCGAAAATGTTGAAAGTATTAGTTA
AAGTGGTTATGCAGTTTTTGCATTTATATATCTGTTAATAGATCAAAAATCAT
CGCTTCGCTGATTAATTACCCCGAGAAATAAGGCTAAAAAACTAATCGCATTAT
CATCCTATGGTTGTTAATTTGATTCGTTTCAATTTGAAGGTTTGTGGGGCCAGGTT
TCTGCTAATTTTTCTTCTTAACCCCCCAAGCTAGTATTGTACAATCTTTAT
TGTTCCGAGCAGTGCGGCGCGAGACGCACCTCCGTTTCCTTTTTCTTTCTTGTG
AAGACGATGACCCCCC}3'

Sample 6: p281-4-p532

Forward amplification:

5'{TGGGGTACTGGCTGGGCTCGGGGACTTTGCGTTCGGGCTGGGAGCGTGCTT
TCCACGACGGTGACACGCTTCCCTGGAATGGCAGCCAGACTGCCTTCCGGGT
CACTGCCATGGAGGAGCCGCAGTCAGATCCTAGCGTCGAGCCCCCTCTGAGT
CAGGAAACATTTTCAGACCTATGGAACTACTTCCTGAAAACAACGTTCTGTC
CCCCTTGCCGTCCCAAGCAGAATTCCCGGGGATCCCGTCGTTTTACAACGTCG
TGACTGGGAAAACCCTGGCGTTACCCAACCTAATCGCCTTGCAGCACATCCCC
CTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCA
ACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTTGCCTGGT}**3'**

Reverse amplification:

5'{GGGAAAAAGGTTGTTTTTCGGAGTAGTTTCCATAGGTCTGAAATGTTTCCTG
ACTCAGAGGGGGCTCGACGCTAGGATCTGACTGCGGCTCCTCCATGGCAGTG
ACCCGGAAGGCAGTCTGGCTGCCAATCCAGGGAAGCGTGTCACCGTCGTGGA
AAGCACGCTCCCAGCCCGAACGCAAAGTGCCCCGGAGCCCAGCAGCTACCT
GCTCCCTGGACGGTGGCTCTAGACCGCGGAGATCCTAGGATCCTAGGATCCT
AGGATCCTAGGATCTTAACAATTTTTTGTGATACTTTTATTACATTTGAATAA
GAAGTAATACAAACCGAAAATGTTGAAAGTATTAGTTAAAGTGGTTATGCAG
TTTTTGCATTTATATATCTGTTAATAGATCAAAAATCATCGCTTCGCTGATTAA
TTACCCCATAAATAAGGCTAAAAAACTAATCGCATTATCATCCTATGGTTGTT
AATTTGATTTCGTTCAATTTGAAGGTTTGCGGGGCCTCGTTCCTGCCT}**3'**

DNA sequencing was performed by Bio Basic, Inc. (Markham, ON, Canada) using the sequence- specific primers listed in Appendix B.

7.4 Appendix D

Results of the large-scale screening

Genes highlighted in green are those found to be positive hits for the corresponding IRES element and one other internal structure. Genes highlighted in yellow are the 7 nonspecific candidate genes identified. Non-highlighted genes represent those specific to each IRES, found through at least 2 of 3 colony lift replicates.

Table D-1 Results of the large-scale screening

FLO8			
GAL1	YBR020W		
HAP3	YBL021C		
FAT1	YBR041W		
ATG8	YBL078C		
BEM1	YBR200W		
HIS7	YBR248C		
CRD1	YDL142C		
BRE1	YDL074C		
UME6	YDR207C		
SIR4	YDR227W		
RMD11	YHL023C		
URA2	YJL130C		
ASF1	YJL115W		
SDS3	YIL084C		
URA1	YKL216W		
URA5	YML106W		
COX5A	YNL052W		
MKS1	YNL076W		
IES2	YNL215W		
YNR018W			
SOL1	YNR034W		
ARG1	YOL058W		
LIP5	YOR196C		
DIA2	YOR080W		
PUT4	YOR348C		
YOR246C			
RPL17B	YJL177W		
YGR273C			
YGR272C			
AXL1	YPR122W		
YMR074C			
KGD2	YDR148C		
HIS2	YFR025C		
ARO2	YGL148W		
NCE102	YPR149W		
ATP20	YPR020W		
AVT2	YEL064C		
RPO41	YFL036W		
GPR1			
AVT2	YEL064C		
WHI3	YNL197C		
ARP8	YOR141C		
VPS5	YOR069W		
ECM33	YBR078W		
SLM3	YDL033C		
RIM1	YCR028C-A		
DBF2	YGR092W		
RIM101	YHL027W		
TRP3	YKL211C		
COX5A	YNL052W		
GCR2	YNL199C		
ATP11	YNL315C		
SLZ1	YNL196C		
STB1	YNL309W		
MKS1	YNL076W		
ATG19	YOL082W		
MMT2	YPL224C	BUB1	YGR188C
ULA1	YPL003W	SNF6	YHL025W
MEP3	YPR138C	FYV10	YIL097W
CCZ1	YBR131W	RPS4A	YJR145C
HAP3	YBL021C	MFT1	YML062C
CSG2	YBR036C	FET3	YMR058W
PRX1	YBL064C	YML013C-A	
RPL19A	YBR084C-A	RPL42A	YNL162W
AVT5	YBL089W	YNL046W	
RTG3	YBL103C	YNR018W	
ATG9	YDL149W	YOL162W	
SNF5	YBR289W	YNR024W	
SUL1	YBR294W	GSH2	YOL049W
GAL3	YDR009W	PHO80	YOL001W
PEX7	YDR142C	HTZ1	YOL012C
RTT103	YDR289C	BTS1	YPL069C
ARG5,6	YER069W	YOR246C	
MET6	YER091C	NCE102	YPR149W
RPL37B	YDR500C	ASN1	YPR145W
PAC10	YGR078C	SNF1	YDR477W
THP2	YHR167W	CDA2	YLR308W

Table C-2 Results of the large-scale screening

YAP1			
RMD11	YHL023C		
YJR054W			
VPS51	YKR020W		
YGR122W			
YMR074C			
NCE102	YPR149W		
YME1	YPR024W		
LSM7	YNL147W		
YDR186C			
VPS41	YDR080W		
MSN5	YDR335W		
VID21	YDR359C		
LSM6	YDR378C		
SOH1	YGL127C		
THP2	YHR167W		
HCR1	YLR192C		
YMR166C			
IES2	YNL215W		
HOS3	YPL116W		
HAP3	YBL021C		
RTG3	YBL103C		
BCS1	YDR375C		
SWI4	YER111C		
HAP2	YGL237C		
CBP4	YGR174C		
AAT1	YKL106W		
VRP1	YLR337C		
LIP2	YLR239C		
COX12	YLR038C	IDH1	YNL037C
SWI6	YLR182W	YNL170W	
GAL2	YLR081W	YNR018W	
TUS1	YLR425W	CYT2	YKL087C
URA4	YLR420W	YGR273C	
FAR3	YMR052W	AXL1	YPR122W
ATG16	YMR159C	ASN1	YPR145W
YML090W		MEP3	YPR138C
PGM2	YMR105C	ERV2	YPR037C
COX5A	YNL052W	CDA2	YLR308W

NCE102	
GAL1	YBR020W
AGP1	YCL025C
MET18	YIL128W
GSH2	YOL049W
CSG2	YBR036C
ATG12	YBR217W
PAT1	YCR077C
PTC1	YDL006W
SLM3	YDL033C
VAM6	YDL077C
SEM1	YDR363W-A
VMA21	YGR105W
LSM1	YJL124C
YJR054W	
YLR261C	
MFT1	YML062C
FET3	YMR058W
YNL140C	
SIN3	YOL004W
BTS1	YPL069C
YGR255C	
CDA2	YLR308W
HAP3	YBL021C
RPL19A	YBR084C-A
BUD14	YAR014C
BCS1	YDR375C
GAL3	YDR009W
PEX7	YDR142C
KGD2	YDR148C
RTT103	YDR289C
CEM1	YER061C
DBF2	YGR092W
SOH1	YGL127C
THP2	YHR167W
SRB2	YHR041C
YML030W	
COX5A	YNL052W
MKS1	YNL076W
LIP5	YOR196C

7.5 Appendix E

Table – E-1: Full list of genes significantly affected, after exposure for Mn²⁺

	ORF	Gene	ave (control)	sd	n	ave (MnCl2)	sd	n	Score (Control-MnCl2)	Sensitivity Intensity (%)
1	YGL168W	HUR1	1.170865	0.036075	3	0.002035	0.002035	3	1.16883	117
2	YGR081C	SLX9	1.152335	0.011215	3	0.197255	0.063595	3	0.95508	98
3	YDR305C	HNT2	1.083995	0.091093	4	0.33684	0.185545	4	0.747155	77
4	YOR069W	VP55	1.208313	0.174882	4	0.4927775	0.173317	4	0.7155355	80
5	YPR119W	CLB2	1.097477	0.052092	3	0.4377133	0.091313	3	0.6597637	70
6	YFR039C	OSW7	1.08166	0.22522	3	0.425725	0.198735	3	0.655935	69
7	YJL088W	ARG3	1.053865	0.017035	3	0.430625	0.016145	3	0.62324	65
8	YOR035C	SHE4	0.90118	0.051217	4	0.3129625	0.047859	4	0.5882175	55
9	YPR046W	MCM16	0.9537067	0.012492	3	0.3797467	0.076557	3	0.57396	56
10	YOL113W	SKM1	1.244925	0.138617	4	0.707675	0.132938	4	0.53725	68
11	YBR164C	ARL1	1.03298	0.017411	4	0.5271025	0.055719	4	0.5058775	52
12	YOR298C-A	MBF1	1.157615	0.040069	4	0.66817	0.099346	4	0.489445	58
13	YPR155C	NCA2	0.90624	0.019924	3	0.5248333	0.042673	3	0.4714067	47
14	YKR023W	YKR023W	1.14421	0.105178	4	0.6748275	0.112328	4	0.4693825	55
15	YJL083W	TAX4	1.09023	0.06004	3	0.66728	0.07136	3	0.42295	48
16	YDL190C	UFD2	1.028383	0.047172	4	0.63136	0.06979	4	0.397023	41
17	YLR420W	URA4	1.458403	0.010124	3	1.067497	0.036561	3	0.390906	73
18	YGR053C	YGR053C	1.075375	0.011815	3	0.696855	0.015395	3	0.37852	43
19	YGR236C	SPG1	1.149022	0.082789	4	0.7781675	0.085497	4	0.3708545	47
20	YHR105W	YPT35	1.09611	0.026874	4	0.7262625	0.043143	4	0.3698475	43
21	YMR209C	YMR209C	1.03769	0.03663	3	0.678735	0.033595	3	0.358955	38
22	YJL160C	PIR5	1.03089	0.00348	3	0.67749	0.02608	3	0.3534	37
23	YOR043W	WH2	0.982135	0.05962	4	0.635275	0.096336	4	0.34686	34
24	YML019W	OST6	1.25041	0.093263	3	0.918	0.074999	3	0.33241	52
25	YOR356W	CIR2	1.09134	0.058099	4	0.7641175	0.097666	4	0.3272225	39
26	YPL259C	APM1	1.088425	0.091109	4	0.7641075	0.116263	4	0.3243175	39
27	YOR196C	LIP5	1.060465	0.043026	4	0.7422575	0.075072	4	0.3182075	36
28	YDR503C	LPP1	1.02099	0.021008	5	0.706006	0.054774	5	0.314984	33
29	YBL031W	SHE1	0.90021	0.061126	4	0.5860325	0.045859	4	0.3141775	25
30	YOR339C	UBC11	1.140852	0.096454	4	0.82858	0.045355	4	0.312272	41
31	YAR002C-A	ERP1	1.075498	0.051323	4	0.76688	0.094286	4	0.308618	36
32	YHR146W	CRP1	1.097638	0.043653	4	0.79051	0.042915	4	0.307128	38
33	YHR130C	YHR130C	1.072762	0.078784	4	0.765925	0.078998	4	0.306837	36
34	YKL092C	BUD2	1.0376	0.100048	4	0.73092	0.019977	4	0.30668	33
35	YDR202C	RAV2	1.028592	0.06063	4	0.7263525	0.0449	4	0.3022395	32
36	YEL039C	CYC7	1.043434	0.034105	5	0.748896	0.068135	5	0.294538	33
37	YBR034C	HMT1	1.032915	0.040322	4	0.7421125	0.120755	4	0.2908025	31
38	YDR506C	GMC1	1.032612	0.034063	5	0.751172	0.082956	5	0.28144	31
39	YBL039C	URA7	0.9790025	0.042451	4	0.700915	0.062075	4	0.2780875	26
40	YEL038W	UTR4	0.993002	0.041155	5	0.722974	0.079207	5	0.270028	26
41	YJR147W	HMS2	1.099748	0.021829	4	0.8342575	0.081935	4	0.2654905	34
42	YBR214W	SDS24	1.049032	0.063572	4	0.7838125	0.077667	4	0.2652195	30
43	YML062C	MFT1	1.362	0.034338	3	1.088283	0.045214	3	0.263717	56
44	YFL011W	HXT10	1.056252	0.027572	5	0.805832	0.080767	5	0.25042	29
45	YNL068C	FKH2	0.9983325	0.050421	4	0.75155	0.03427	4	0.2467825	25
46	YPL116W	HOS3	1.164265	0.053946	4	0.9195375	0.032581	4	0.2447275	37
47	YBR200W	BEM1	0.7616675	0.076916	4	0.5195475	0.084815	4	0.24212	8
48	YBR036C	CSG2	1.058998	0.031558	4	0.82043	0.02785	4	0.238568	28
49	YBR073W	RDH54	1.033402	0.041444	4	0.8039275	0.04389	4	0.2294745	26
50	YER065C	ICL1	1.11932	0.064358	5	0.891106	0.050358	5	0.228214	32
51	YER056C	FCY2	1.07875	0.035131	5	0.858666	0.050824	5	0.220084	28
52	YER067C-A	YER067C-A	1.054336	0.036081	5	0.843586	0.059283	5	0.21075	25
53	YDR431W	YDR431W	1.140604	0.054012	5	0.930166	0.021056	5	0.210438	33
54	YDR216W	ADR1	1.099082	0.027995	4	0.8904775	0.071701	4	0.2086045	29
55	YOR120W	GCY1	1.01461	0.023321	4	0.818365	0.062186	4	0.196245	21
56	YDR445C	YDR445C	1.001802	0.026957	5	0.81015	0.062413	5	0.191652	19
57	YHR104W	GRE3	1.029137	0.017892	4	0.8382225	0.049999	4	0.1909145	21
58	YBR129C	OPY1	1.034863	0.038183	4	0.852305	0.015116	4	0.182558	21
59	YAL020C	ATS1	0.9270175	0.039382	4	0.7445325	0.027469	4	0.182485	12
60	YDR097C	MSH6	1.025058	0.039329	4	0.843625	0.015504	4	0.181433	20
61	YML009c	MRPL39	1.058793	0.051166	3	0.88467	0.019255	3	0.174123	22
62	YHL031C	GOS1	0.960635	0.045899	4	0.7893075	0.036217	4	0.1713275	14
63	YOL155C	HPF1	1.013653	0.110044	4	0.844445	0.09764	4	0.169208	18
64	YBL085W	BOI1	1.155473	0.077384	4	0.9927825	0.095165	4	0.1626905	30
65	YBR050C	REG2	1.041288	0.043482	4	0.915575	0.061218	4	0.125713	16
66	YOR347C	PKY2	0.9492275	0.029227	4	0.8269225	0.036802	4	0.122305	8
67	YER151C	UBP3	0.951364	0.01368	5	0.830952	0.027866	5	0.120412	8
68	YBL095W	MRO3	1.018275	0.04173	4	0.9388825	0.034991	4	0.0793925	10

Table E-2: Gene Ontology Analysis (Cellular Component)

#pathway ID	pathway description	Number of observed genes	FDR
GO.0008250	oligosaccharyltransferase complex	7	1.62E-09
GO.0005905	coated pit	6	3.15E-06
GO.0030125	clathrin vesicle coat	6	5.16E-06
GO.0030130	clathrin coat of trans-Golgi network vesicle	5	1.95E-05
GO.0030665	clathrin-coated vesicle membrane	6	2.40E-05
GO.0000817	COMA complex	4	2.91E-05
GO.0012510	trans-Golgi network transport vesicle membrane	5	2.91E-05
GO.0000777	condensed chromosome kinetochore	9	5.69E-05
GO.0030135	coated vesicle	10	6.18E-05
GO.0000778	condensed nuclear chromosome kinetochore	8	6.72E-05
GO.0005656	nuclear pre-replicative complex	6	6.72E-05
GO.0030121	AP-1 adaptor complex	4	6.98E-05
GO.0000779	condensed chromosome centromeric region	9	9.48E-05
GO.0030133	transport vesicle	9	1.22E-04
GO.0000780	condensed nuclear chromosome centromeric region	8	1.80E-04
GO.0030136	clathrin-coated vesicle	6	1.83E-04
GO.0044454	nuclear chromosome part	17	1.85E-04
GO.0031261	DNA replication preinitiation complex	6	2.13E-04
GO.1902494	catalytic complex	25	2.13E-04
GO.0030140	trans-Golgi network transport vesicle	5	2.86E-04
GO.0030906	retromer cargo-selective complex	3	3.53E-04
GO.0034098	VCP-NPL4-UFD1 AAA ATPase complex	3	3.53E-04
GO.0098687	chromosomal region	12	3.82E-04
GO.0000776	kinetochore	8	4.81E-04
GO.0000228	nuclear chromosome	17	5.30E-04
GO.0031298	replication fork protection complex	6	5.58E-04
GO.0000775	chromosome centromeric region	9	6.53E-04
GO.1990234	transferase complex	19	7.60E-04
GO.0005680	anaphase-promoting complex	4	9.48E-04
GO.0000151	ubiquitin ligase complex	8	9.82E-04
GO.0030132	clathrin coat of coated pit	3	2.08E-03
GO.0000793	condensed chromosome	9	2.36E-03
GO.0044427	chromosomal part	17	2.92E-03
GO.0005694	chromosome	18	3.15E-03
GO.1990112	RQC complex	3	3.74E-03
GO.0098796	membrane protein complex	15	4.12E-03
GO.0043596	nuclear replication fork	6	5.24E-03
GO.0000837	Doa10p ubiquitin ligase complex	3	5.68E-03
GO.0030131	clathrin adaptor complex	3	5.68E-03
GO.0000794	condensed nuclear chromosome	8	5.96E-03
GO.0005951	carbamoyl-phosphate synthase complex	2	7.09E-03
GO.0009514	glyoxysome	2	7.09E-03
GO.0030904	retromer complex	2	7.09E-03
GO.0030905	retromer tubulation complex	2	7.09E-03
GO.0044446	intracellular organelle part	73	7.37E-03
GO.0030118	clathrin coat	3	1.07E-02
GO.0005935	cellular bud neck	11	1.25E-02
GO.0070013	intracellular organelle lumen	34	1.25E-02
GO.0031981	nuclear lumen	27	1.27E-02
GO.0005737	cytoplasm	94	1.30E-02
GO.0000839	Hrd1p ubiquitin ligase ERAD-L complex	3	1.32E-02
GO.0000942	condensed nuclear chromosome outer kinetochore	2	1.44E-02
GO.0030122	AP-2 adaptor complex	2	1.44E-02
GO.0030128	clathrin coat of endocytic vesicle	2	1.44E-02
GO.0030669	clathrin-coated endocytic vesicle membrane	2	1.44E-02
GO.0030869	RENT complex	2	1.44E-02
GO.0031974	membrane-enclosed lumen	35	1.44E-02
GO.0036266	Cdc48p-Npl4p-Vms1p AAA ATPase complex	2	1.44E-02
GO.0043234	protein complex	40	1.44E-02
GO.0043291	RAVE complex	2	1.44E-02
GO.0045334	clathrin-coated endocytic vesicle	2	1.44E-02
GO.0097373	MCM core complex	2	1.44E-02
GO.0000139	Golgi membrane	9	2.58E-02
GO.0012505	endomembrane system	29	2.58E-02
GO.0043231	intracellular membrane-bounded organelle	91	2.67E-02
GO.0030427	site of polarized growth	13	2.91E-02
GO.0098588	bounding membrane of organelle	28	3.19E-02
GO.0044424	intracellular part	99	3.63E-02
GO.0032221	Rpd3S complex	2	4.14E-02
GO.0005819	spindle	7	4.74E-02
GO.0030134	ER to Golgi transport vesicle	4	4.74E-02
GO.0000307	cyclin-dependent protein kinase holoenzyme complex	3	4.81E-02
GO.0043229	intracellular organelle	91	4.92E-02

Table E-3: Gene Ontology Analysis (Molecular Function)

#pathway ID	pathway description	Number of observed genes	FDR
GO.0004579	dolichyl-diphosphooligosaccharide-protein glycotransferase activity	5	7.98E-06
GO.0004576	oligosaccharyl transferase activity	4	1.92E-04
GO.0008565	protein transporter activity	7	6.11E-03
GO.0016538	cyclin-dependent protein serine/threonine kinase regulator activity	6	6.11E-03
GO.0030276	clathrin binding	4	3.32E-02
GO.0003678	DNA helicase activity	5	4.89E-02
GO.0030234	enzyme regulator activity	14	4.89E-02
GO.0032041	NAD-dependent histone deacetylase activity (H3-K14 specific)	3	4.89E-02

Table E-4: Gene Ontology Analysis (Biological Processes)

#pathway ID	pathway description	Number of observed genes	FDR
GO.0000278	mitotic cell cycle	40	3.04E-16
GO.1903047	mitotic cell cycle process	39	6.45E-16
GO.0051301	cell division	35	7.24E-15
GO.0007049	cell cycle	51	1.88E-14
GO.0022402	cell cycle process	46	2.88E-13
GO.0007067	mitotic nuclear division	26	2.07E-12
GO.0000280	nuclear division	30	3.83E-11
GO.0051726	regulation of cell cycle	26	6.44E-11
GO.0044772	mitotic cell cycle phase transition	16	3.58E-09
GO.0000079	regulation of cyclin-dependent protein serine/threonine kinase activity	10	2.22E-08
GO.0010695	regulation of spindle pole body separation	7	2.22E-08
GO.1904029	regulation of cyclin-dependent protein kinase activity	11	7.15E-08
GO.0044699	single-organism process	88	9.91E-08
GO.0010564	regulation of cell cycle process	19	2.34E-07
GO.0051321	meiotic cell cycle	25	2.61E-07
GO.0051338	regulation of transferase activity	15	3.31E-07
GO.0007346	regulation of mitotic cell cycle	16	3.34E-07
GO.0051302	regulation of cell division	16	1.01E-06
GO.0033043	regulation of organelle organization	24	1.02E-06
GO.0000086	G2/M transition of mitotic cell cycle	9	1.85E-06
GO.0034087	establishment of mitotic sister chromatid cohesion	7	1.85E-06
GO.0090068	positive regulation of cell cycle process	10	1.85E-06
GO.0033044	regulation of chromosome organization	15	2.05E-06
GO.0051128	regulation of cellular component organization	27	3.72E-06
GO.0007088	regulation of mitotic nuclear division	12	4.60E-06
GO.0031399	regulation of protein modification process	16	1.07E-05
GO.0006591	ornithine metabolic process	5	1.13E-05
GO.0010696	positive regulation of spindle pole body separation	5	1.13E-05
GO.0030071	regulation of mitotic metaphase/anaphase transition	9	1.13E-05
GO.0051783	regulation of nuclear division	14	1.13E-05
GO.0033260	nuclear DNA replication	8	1.75E-05
GO.0051983	regulation of chromosome segregation	10	1.88E-05
GO.0000003	reproduction	28	1.96E-05
GO.0070507	regulation of microtubule cytoskeleton organization	8	2.62E-05
GO.0098813	nuclear chromosome segregation	13	3.05E-05
GO.0044763	single-organism cellular process	78	3.58E-05
GO.1901990	regulation of mitotic cell cycle phase transition	11	4.32E-05
GO.2001251	negative regulation of chromosome organization	11	4.85E-05
GO.0051439	regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	7	5.46E-05
GO.0051129	negative regulation of cellular component organization	14	6.64E-05
GO.0010965	regulation of mitotic sister chromatid separation	8	7.02E-05
GO.0045859	regulation of protein kinase activity	10	8.02E-05
GO.0065007	biological regulation	57	1.07E-04
GO.0051340	regulation of ligase activity	7	1.11E-04
GO.0031396	regulation of protein ubiquitination	8	1.16E-04
GO.0031329	regulation of cellular catabolic process	12	1.22E-04
GO.0006275	regulation of DNA replication	9	1.42E-04
GO.0050790	regulation of catalytic activity	20	1.42E-04
GO.0000226	microtubule cytoskeleton organization	11	1.57E-04
GO.0010639	negative regulation of organelle organization	13	1.66E-04
GO.0006267	pre-replicative complex assembly involved in nuclear cell cycle DNA replication	6	1.70E-04
GO.0006526	arginine biosynthetic process	4	1.83E-04
GO.0051230	spindle disassembly	4	1.83E-04
GO.0050789	regulation of biological process	51	1.96E-04
GO.0000070	mitotic sister chromatid segregation	9	2.19E-04
GO.0000819	sister chromatid segregation	10	2.19E-04
GO.0045786	negative regulation of cell cycle	11	2.32E-04
GO.0006487	protein N-linked glycosylation	7	2.68E-04
GO.0010948	negative regulation of cell cycle process	10	2.68E-04
GO.0051781	positive regulation of cell division	5	2.68E-04
GO.0007051	spindle organization	7	3.20E-04
GO.0051985	negative regulation of chromosome segregation	7	3.84E-04
GO.0000212	meiotic spindle organization	3	4.47E-04
GO.0007064	mitotic sister chromatid cohesion	7	4.47E-04
GO.0007092	activation of APC-Cdc20 complex activity	3	4.47E-04
GO.0018279	protein N-linked glycosylation via asparagine	3	4.47E-04
GO.0051052	regulation of DNA metabolic process	11	4.96E-04
GO.0000082	G1/S transition of mitotic cell cycle	8	5.52E-04
GO.0030970	retrograde protein transport ER to cytosol	4	5.78E-04
GO.0031398	positive regulation of protein ubiquitination	4	5.78E-04
GO.0007059	chromosome segregation	13	6.32E-04
GO.0048522	positive regulation of cellular process	27	7.17E-04
GO.0080090	regulation of primary metabolic process	38	7.34E-04

... Continuation of Table E4: Gene Ontology Analysis (Biological Processes)

#pathway ID	pathway description	Number of observed genes	FDR
GO.0010638	positive regulation of organelle organization	10	8.20E-04
GO.0009084	glutamine family amino acid biosynthetic process	5	8.82E-04
GO.0032880	regulation of protein localization	9	9.06E-04
GO.1903046	meiotic cell cycle process	17	9.06E-04
GO.0031323	regulation of cellular metabolic process	38	9.22E-04
GO.0032507	maintenance of protein location in cell	6	1.10E-03
GO.0050794	regulation of cellular process	46	1.10E-03
GO.0060255	regulation of macromolecule metabolic process	37	1.11E-03
GO.0045930	negative regulation of mitotic cell cycle	8	1.24E-03
GO.0043086	negative regulation of catalytic activity	9	1.31E-03
GO.0007010	cytoskeleton organization	14	1.32E-03
GO.0006592	ornithine biosynthetic process	3	1.42E-03
GO.0070727	cellular macromolecule localization	23	1.57E-03
GO.0032268	regulation of cellular protein metabolic process	18	1.64E-03
GO.0030433	ER-associated ubiquitin-dependent protein catabolic process	7	1.70E-03
GO.0036503	ERAD pathway	7	1.70E-03
GO.0007094	mitotic spindle assembly checkpoint	5	1.75E-03
GO.0008608	attachment of spindle microtubules to kinetochore	5	1.75E-03
GO.0006525	arginine metabolic process	4	1.94E-03
GO.0019222	regulation of metabolic process	40	1.94E-03
GO.0034613	cellular protein localization	22	1.94E-03
GO.0044710	single-organism metabolic process	44	1.94E-03
GO.0071174	mitotic spindle checkpoint	6	1.94E-03
GO.0031400	negative regulation of protein modification process	8	2.09E-03
GO.0031330	negative regulation of cellular catabolic process	7	2.23E-03
GO.0071712	ER-associated misfolded protein catabolic process	3	2.77E-03
GO.0051495	positive regulation of cytoskeleton organization	6	2.93E-03
GO.0007093	mitotic cell cycle checkpoint	7	3.09E-03
GO.0048518	positive regulation of biological process	28	3.15E-03
GO.1903827	regulation of cellular protein localization	6	3.27E-03
GO.0051348	negative regulation of transferase activity	7	3.35E-03
GO.0045740	positive regulation of DNA replication	4	3.42E-03
GO.0033036	macromolecule localization	30	3.51E-03
GO.0000075	cell cycle checkpoint	8	3.64E-03
GO.0090329	regulation of DNA-dependent DNA replication	6	3.64E-03
GO.0043161	proteasome-mediated ubiquitin-dependent protein catabolic process	10	3.69E-03
GO.0048519	negative regulation of biological process	25	4.13E-03
GO.0044205	de novo UMP biosynthetic process	3	4.68E-03
GO.0070932	histone H3 deacetylation	3	4.68E-03
GO.0000727	double-strand break repair via break-induced replication	5	4.86E-03
GO.0008104	protein localization	26	5.13E-03
GO.0045931	positive regulation of mitotic cell cycle	4	7.30E-03
GO.0051984	positive regulation of chromosome segregation	4	7.30E-03
GO.0006122	mitochondrial electron transport ubiquinol to cytochrome c	3	7.50E-03
GO.0006621	protein retention in ER lumen	3	7.50E-03
GO.1900182	positive regulation of protein localization to nucleus	3	7.50E-03
GO.1990116	ribosome-associated ubiquitin-dependent protein catabolic process	3	7.50E-03
GO.1902589	single-organism organelle organization	34	7.91E-03
GO.0006281	DNA repair	13	8.77E-03
GO.0032269	negative regulation of cellular protein metabolic process	9	8.85E-03
GO.0051054	positive regulation of DNA metabolic process	5	8.91E-03
GO.0051784	negative regulation of nuclear division	7	9.96E-03
GO.0006527	arginine catabolic process	2	1.02E-02
GO.0010697	negative regulation of spindle pole body separation	2	1.02E-02
GO.0034089	establishment of meiotic sister chromatid cohesion	2	1.02E-02
GO.0051229	meiotic spindle disassembly	2	1.02E-02
GO.1903829	positive regulation of cellular protein localization	4	1.08E-02
GO.0071629	cytoplasm-associated proteasomal ubiquitin-dependent protein catabolic process	3	1.10E-02
GO.0051347	positive regulation of transferase activity	5	1.15E-02
GO.0007126	meiotic nuclear division	10	1.21E-02
GO.0009892	negative regulation of metabolic process	20	1.55E-02
GO.0031145	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	3	1.55E-02
GO.0031331	positive regulation of cellular catabolic process	5	1.70E-02
GO.0030174	regulation of DNA-dependent DNA replication initiation	4	1.88E-02
GO.0045333	cellular respiration	7	2.14E-02
GO.0006302	double-strand break repair	8	2.28E-02
GO.0006896	Golgi to vacuole transport	4	2.62E-02
GO.0034090	maintenance of meiotic sister chromatid cohesion	2	2.66E-02
GO.0035247	peptidyl-arginine omega-N-methylation	2	2.66E-02
GO.0070933	histone H4 deacetylation	2	2.66E-02
GO.0072671	mitochondria-associated ubiquitin-dependent protein catabolic process	2	2.66E-02
GO.0045053	protein retention in Golgi apparatus	3	2.71E-02
GO.0015031	protein transport	21	2.77E-02
GO.0000083	regulation of transcription involved in G1/S transition of mitotic cell cycle	4	2.95E-02

... Continuation of Table E-4: Gene Ontology Analysis (Biological Processes)

#pathway ID	pathway description	Number of observed genes	FDR
GO.0010605	negative regulation of macromolecule metabolic process	18	3.14E-02
GO.0000725	recombinational repair	6	3.38E-02
GO.0090224	regulation of spindle organization	3	3.38E-02
GO.2001252	positive regulation of chromosome organization	4	3.38E-02
GO.0006486	protein glycosylation	5	3.44E-02
GO.0031401	positive regulation of protein modification process	6	4.15E-02
GO.0071218	cellular response to misfolded protein	3	4.15E-02
GO.0031938	regulation of chromatin silencing at telomere	4	4.24E-02
GO.0006207	de novo pyrimidine nucleobase biosynthetic process	2	4.58E-02
GO.0008054	negative regulation of cyclin-dependent protein serine/threonine kinase by cyclin degradation	2	4.58E-02
GO.0009249	protein lipoylation	2	4.58E-02
GO.0045736	negative regulation of cyclin-dependent protein serine/threonine kinase activity	2	4.58E-02
GO.0051228	mitotic spindle disassembly	2	4.58E-02
GO.0044257	cellular protein catabolic process	11	4.64E-02
GO.0007052	mitotic spindle organization	4	4.70E-02
GO.0009064	glutamine family amino acid metabolic process	5	4.80E-02

Table E-5: Gene Ontology Analysis (Pathways)

#pathway ID	pathway description	Number of observed genes	FDR
4111	Cell cycle - yeast	29	8.46E-21
4113	Meiosis - yeast	21	1.26E-11
4141	Protein processing in endoplasmic reticulum	17	1.78E-11
513	Various types of N-glycan biosynthesis	9	1.89E-07
510	N-Glycan biosynthesis	9	2.09E-07
1100	Metabolic pathways	36	2.83E-06
330	Arginine and proline metabolism	8	2.84E-06
785	Lipoic acid metabolism	3	1.28E-04
4120	Ubiquitin mediated proteolysis	7	6.42E-04
250	Alanine aspartate and glutamate metabolism	5	4.12E-03
3030	DNA replication	5	4.39E-03
630	Glyoxylate and dicarboxylate metabolism	4	9.66E-03
1230	Biosynthesis of amino acids	8	3.47E-02
1210	2-Oxocarboxylic acid metabolism	4	4.31E-02

7.6 Appendix F

Figure F-1: The relative expression of β -galactosidase in response to the presence on increasing concentrations (0 – 5 mM) of ions Ca^{2+} , Mg^{2+} and Zn^{2+} . In the specific case of the Mg^{2+} , the higher concentration was 15 mM.

Figure F-2: Humans and yeast share orthologous genes, potentially linked to neurodegenerative disorders. Approximately 31% (44 proteins/genes) of the inferred network for hits (143 proteins/genes) affected by manganese have homologues in human (A), of which approximately 22% (32 proteins/genes) are potentially associated with neurodegeneration (B).

7.8 Appendix G

Determination of minimum inhibitory concentrations (MICs)

Dilution methods are used to determine the minimum inhibitory concentrations (MICs) of all chemicals used for yeast sensitivity. Microdilution panels were used for MIC determination that aids in selection of appropriate sub-inhibitory concentrations for different test. Strong sub-inhibitory concentrations (determined manually) were used for drug sensitivity analysis of susceptible mutants to better highlight sensitivity. Weak sub-inhibitory (arbitrarily selected) concentrations were used for conditional GI analysis.

In this test yeast cells are tested for their ability to produce visible growth on a series of dilutions containing multiple concentrations of the tested chemical reagents. The lowest concentration of a reagent that prevents the appearance of visible growth of the yeast cells within a defined period of time, is known as the MIC.

During testing, multiple wells in microplates are filled with a growth medium (e.g. YPD or YNB). Varying concentrations of the tested chemicals and the yeast cells to be tested are then added to the wells (Fig G-1). The plate(s) is then placed into an incubator/shaker at 30°C and grown over night. The next day, the plate is removed and checked for bacterial growth. If the media became cloudy or a layer of cells formed at the bottom of the wells (Fig G-1). The results of the liquid microdilution method are collected in MIC, or the lowest concentration of the chemical that stopped the yeast expansion.

Figure G-1: representation of a microdilution panel. Custom panels are created using a 96-well format plates.

7.7 Appendix H

List of publications

This chapter is published in *PLoS One* journal

Chapter 2: 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

Houman Moteshareie, Maryam Hajikarimlou, Alex Mulet Indrayanti, Daniel Burnside, Ana Paula Dias, Clara Lettl, Duale Ahmed, Katayoun Omid, Tom Kazmirchuk, Nathalie Puchacz, Narges Zare, Sarah Takallou, Thet Naing, Raúl Bonne Hernández, William G. Willmore, Mohan Babu, Bruce McKay, Bahram Samanfar, Martin Holcik, Ashkan Golshani

Published in *PLoS One*

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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

Houman Moteshareie, Maryam Hajikarimlou, Alex Mulet Indrayanti, Daniel Burnside, Ana Paula Dias, Clara Lettl, Duale Ahmed, Katayoun Omid, Tom Kazmirchuk, Nathalie Puchacz, Narges Zare, Sarah Takallou, Thet Naing, [...], Ashkan Golshani
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Published: September 19, 2018 • <https://doi.org/10.1371/journal.pone.0198704> • >> See the preprint

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Abstract

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Materials and methods

Results and discussion

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References

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Figures

Abstract

Heavy metal and metalloid contaminations are among the most concerning types of pollutant in the environment. Consequently, it is important to investigate the molecular mechanisms of cellular responses and detoxification pathways for these compounds in living organisms. To date, a number of genes have been linked to the detoxification process. The expression of these genes can be controlled at both transcriptional and translational levels. In baker's yeast, *Saccharomyces cerevisiae*, resistance to a wide range of toxic metals is regulated by glutathione S-transferases. Yeast *URE2* encodes for a protein that has glutathione peroxidase activity and is homologous to mammalian glutathione S-transferases. The *URE2* expression is critical to cell survival under heavy metal stress. Here, we report on the finding of two genes, *ITT1*, an inhibitor of translation termination, and *RPS1A*, a small ribosomal protein, that when deleted yeast cells exhibit similar metal sensitivity phenotypes to gene deletion strain for *URE2*. Neither of these genes were previously linked to metal toxicity. Our gene expression analysis illustrates that these two genes affect *URE2* mRNA expression at the level of translation.

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A Screen for Candidate Targets of Lysine Polyphosphorylation Uncovers a Conserved Network Implicated in Ribosome Biogenesis.

Bentley-DeSousa A, **Moteshareie H***, Holinier C*, Tseng YC, Kajjo S, Nwosu C, Amodeo GF, Bondy-Chorney E, Sai Y, Rudner A, Golshani A, Davey NE, Downey M.

* equally contributed

Published in *Cell reports*

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A Screen for Candidate Targets of Lysine Polyphosphorylation Uncovers a Conserved Network Implicated in Ribosome Biogenesis

Amanda Bentley-DeSousa • Charlotte Holinier [†] • Hourman Moleshareio [‡] • Yi-Chieh Tseng • Sam Kajjo •
 Christine Nwosu • Giuseppe Federico Amodeo • Emma Bondy-Chorney • Yuka Sai • Adam Rudner •
 Ashkan Golshani • Norman E. Davey • Michael Downey [✉] [§] [Show less](#) [Show footnotes](#)

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Highlights

- A strategy for identifying targets of lysine polyphosphorylation
- 15 yeast targets include a conserved network involved in ribosome biogenesis
- Defects in polyP synthesis result in phenotypes related to translation

The sensitivity of the yeast, *Saccharomyces cerevisiae*, to acetic acid is influenced by DOM34 and RPL36A

Samanfar B, Shostak K, **Moteshareie H**, Hajikarimlou M, Shaikho S, Omid K, Hooshyar M, Burnside D, Márquez IG, Kazmirchuk T, Naing T, Ludovico P, York-Lyon A, Szereszewski K, Leung C, Jin JY, Megarbane R, Smith ML, Babu M, Holcik M, Golshani A.

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The sensitivity of the yeast, *Saccharomyces cerevisiae*, to acetic acid is influenced by *DOM34* and *RPL36A*

Research article Cell Biology Genetics Molecular Biology

Bahram Samanfar^{1,2}, Kristina Shostak^{1,2}, Houman Moteshareie¹, Maryam Hajikarimlou¹, Sarah Shaikho³, Katayoun Omid K¹, Mohsen Hooshyar^{1,4}, Daniel Burnside¹, Imelda Galván Márquez¹, Tom Kazmirchuk¹, Thet Naing³, Paula Ludovico⁵, Anna York-Lyon^{*1}, Kama Szereszewski^{*1,6}, Cindy Leung^{*1}, Jennifer Yixin Jin^{*1}, Rami Megarbane^{*1}, Myron L. Smith¹, Mohan Babu⁷, Martin Holcik^{3,8}, Ashkan Golshani^{✉1}

Published November 14, 2017 PubMed 29158977

> Author and article information

▼ Abstract

The presence of acetic acid during industrial alcohol fermentation reduces the yield of fermentation by imposing additional stress on the yeast cells. The biology of cellular responses to stress has been a subject of vigorous

Evolution of protein-protein interaction networks in yeast

Schoenrock, A., Burnside, D., **Moteshareie, H.**, Pitre, S., Hooshyar, M., Green, J.R., Golshani, A., Dehne, F. and Wong, A.

Published in *PloS one*

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Evolution of protein-protein interaction networks in yeast

Andrew Schoenrock, Daniel Burnside, Houman Moteshareie, Sylvain Pitre, Mohsen Hooshyar, James R. Green, Ashkan Golshani, Frank Dehne, Alex Wong

Published: March 1, 2017 • <https://doi.org/10.1371/journal.pone.0171920>

Article	Authors	Metrics	Comments	Media Coverage

Abstract

Introduction

Materials and methods

Results

Discussion

Conclusions

Supporting information

Acknowledgments

Author Contributions

Abstract

Interest in the evolution of protein-protein and genetic interaction networks has been rising in recent years, but the lack of large-scale high quality comparative datasets has acted as a barrier. Here, we carried out a comparative analysis of computationally predicted protein-protein interaction (PPI) networks from five closely related yeast species. We used the Protein-protein Interaction Prediction Engine (PIPE), which uses a database of known interactions to make sequence-based PPI predictions, to generate high quality predicted interactomes. Simulated proteomes and corresponding PPI networks were used to provide null expectations for the extent and nature of PPI network evolution. We found strong evidence for conservation of PPIs, with lower than expected levels of change in PPIs for about a quarter of the proteome. Furthermore, we found that changes in predicted PPI networks are poorly predicted by sequence divergence. Our analyses identified a number of functional classes experiencing

The genome of an *Encephalitozoon cuniculi* type III strain reveals insights into the genetic diversity and mode of reproduction of a ubiquitous vertebrate pathogen

Pelin, A., Moteshareie, H., Sak, B., Selman, M., Naor, A., Eyahpaise, M.È., Farinelli, L., Golshani, A., Kvac, M. and Corradi, N.

Published in *Heredity*

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Original Article | Published: 03 February 2016

The genome of an *Encephalitozoon cuniculi* type III strain reveals insights into the genetic diversity and mode of reproduction of a ubiquitous vertebrate pathogen

A Pelin, H Moteshareie, B Sak, M Selman, A Naor, M-È Eyahpaise, L Farinelli, A Golshani, M Kvac & N Corradi ✉

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Abstract

Encephalitozoon cuniculi is a model microsporidian species with a mononucleate nucleus and a genome that has been extensively studied. To date, analyses of genome diversity have revealed the existence of four genotypes in *E. cuniculi* (EcI, II, III and IV). Genome sequences are available for EcI, II and III, and are all very divergent, possibly diploid and genetically homogeneous. The mechanisms that cause low genetic diversity in *E. cuniculi* (for example, selfing, inbreeding or a

Elevated levels of ribosomal proteins eL36 and eL42 control expression of Hsp90 in rhabdomyosarcoma

Shaikho, S., Dobson, C.C., Naing, T., Samanfar, B., **Moteshareie, H.**, Hajikarimloo, M., Golshani, A. and Holcik, M.

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Elevated levels of ribosomal proteins eL36 and eL42 control expression of Hsp90 in rhabdomyosarcoma

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Schoenrock, A., Burnside, D., **Moteshareie, H.**, Wong, A., Golshani, A., Dehne, F. and Green, J.R.

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<div>Document Sections</div> <div><div>1. Introduction</div><div>2. In-Silico Protein Synthesizer (INSIPS)</div><div>3. Performance Evaluation</div><div>4. Experimental Validation</div></div>	<p>Engineered proteins are synthetic novel proteins (not found in nature) that are designed to fulfill a predetermined biological function. Such proteins can be used as molecular markers, inhibitory agents, or drugs. For example, a synthetic protein could bind to a critical protein of a pathogen, thereby inhibiting the function of the target protein and potentially reducing the impact of the pathogen. In this paper we present the In-Silico Protein Synthesizer (InSiPS), a massively parallel computational tool for the IBM Blue Gene/Q that is aimed at designing inhibitory proteins. More precisely, InSiPS designs proteins that are predicted to interact with a given target protein (and may inhibit the target's cellular functions) while leaving non-target proteins unaffected (to minimize side-effects). As proof-of-concepts, two InSiPS designed proteins have been synthesized in the lab and their inhibitory properties have been experimentally verified through wet-lab experimentation.</p>

Designing anti-Zika virus peptides derived from predicted human-Zika virus protein-protein interactions

Kazmirchuk, T., Dick, K., Burnside, D.J., Barnes, B., **Moteshareie, H.**, Hajikarimlou, M., Omid, K., Ahmed, D., Low, A., Lettl, C. and Hooshyar, M.

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Designing anti-Zika virus peptides derived from predicted human-Zika virus protein-protein interactions

Tom Kazmirchuk ^a, Kevin Dick ^b, Daniel. J. Burnside ^a, Brad Barnes ^b, Houman Moteshareie ^a, Maryam Hajikarimlou ^a, Katayoun Omid ^a, Duale Ahmed ^{a, c}, Andrew Low ^a, Clara Lettl ^a, Mohsen Hooshyar ^a, Andrew Schoenrock ^d, Sylvain Pitre ^d, Mohan Babu ^a, Edana Cassol ^e, Bahram Samanfar ^f, Alex Wong ^a, Frank Dehne ^c, James. R. Green ^b, Ashkan Golshani ^a

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Uncharacterized ORF HUR1 influences the efficiency of non-homologous end-joining repair in *Saccharomyces cerevisiae*

Omidi, K., Jessulat, M., Hooshyar, M., Burnside, D., Schoenrock, A., Kazmirchuk, T., Hajikarimlou, M., Daniel, M., **Moteshareie, H.**, Bhojoo, U. and Sanders, M.

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Research paper

Uncharacterized ORF *HUR1* influences the efficiency of non-homologous end-joining repair in *Saccharomyces cerevisiae*

Katayoun Omidi ^{a, b}, Matthew Jessulat ^c, Mohsen Hooshyar ^{a, b}, Daniel Burnside ^{a, b}, Andrew Schoenrock ^d, Tom Kazmirchuk ^{a, b}, Maryam Hajikarimlou ^{a, b}, Mary Daniel ^{a, b}, Houman Moteshareie ^{a, b}, Urvi Bhojoo ^{a, b}, Megan Sanders ^{a, b}, Dindial Ramotar ^e, Frank Dehne ^d, Bahram Samanfar ^f, Mohan Babu ^c, Ashkan Golshani ^{a, b}

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