Investigating Cellular Stress and Cellular Response Relationships in Yeast

David Zhou

Degree of Doctor of Philosophy

Department of Anatomy & Cell Biology,

McGill University

Montreal, Quebec, Canada

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

August 2022

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Abstract

Throughout a cell's lifetime, it will experience changes to its external and/or internal environment that have the potential to perturb its own homeostasis. These challenges are defined as stress and the cellular response to it can vary. Traditionally, this relationship has been modelled as a continuum where the intensity of stress dictates the cellular response. In this thesis, the relationship between cellular stress and response at different stress intensities is explored using the yeast *Saccharomyces cerevisiae*. Using both growth and viability assays, we show that this continuum is indeed malleable and the point at which cells undergo RCD in response to stress can be delayed by the heterologous expression of pro-survival sequences. These pro-survival sequences are also useful as tools to screen against different stresses and elucidate novel stress-dependent pathways. In this way, we demonstrate that the cellular response to excess iron and copper stress is distinct from one another and share some degree of crosstalk. Furthermore, this thesis re-examines the relationship between extreme stress intensities and its effects on cellular viability. Interestingly, stress intensity does not always faithfully predict the cellular response. Indeed, when cells are subjected to extreme levels of certain RCD-inducing stress including excess copper and lithium, necrosis is unachievable with intensity alone. These findings contrast the traditional model in which we relate cellular stress to cellular response. Investigating a newer model for cellular stress vs response relationships is both an interesting and important area for future cellular stress research.

Resumé

Tout au long de sa vie, une cellule connaîtra des changements dans son environnement externe et/ou interne susceptibles de perturber sa propre homéostasie. Ces défis sont définis comme du stress et la réponse cellulaire à celui-ci peut varier. Traditionnellement, cette relation a été modélisée comme un continuum où l'intensité du stress dicte la réponse cellulaire. Dans cette thèse, la relation entre le stress cellulaire et la réponse à différentes intensités de stress est explorée en utilisant la levure *Saccharomyces cerevisiae*. En utilisant à la fois des tests de croissance et de viabilité, nous montrons que ce continuum est en effet malléable et que le point auquel les cellules subissent une RCD en réponse au stress peut être retardé par l'expression hétérologue de séquences pro-survie. Ces séquences pro-survie sont également utiles comme outils pour dépister différents stress et élucider de nouvelles voies dépendantes du stress. De cette façon, nous démontrons que la réponse cellulaire à un stress excessif en fer et en cuivre est distincte l'une de l'autre et partage un certain degré de diaphonie. De plus, cette thèse réexamine la relation entre les intensités de stress extrême et ses effets sur la viabilité cellulaire. Fait intéressant, l'intensité du stress ne prédit pas toujours fidèlement la réponse cellulaire. En effet, lorsque les cellules sont soumises à des niveaux extrêmes de certaines contraintes induisant des RCD, notamment un excès de cuivre et de lithium, la nécrose est impossible à atteindre avec la seule intensité. Ces résultats contrastent avec le modèle traditionnel dans lequel nous relions le stress cellulaire à la réponse cellulaire. L'étude d'un modèle plus récent pour les relations entre le stress cellulaire et la réponse est à la fois un domaine intéressant et important pour la recherche future sur le stress cellulaire.

Acknowledgements

Firstly, thank you to my supervisors Dr Greenwood and Dr Mandato. You have each given me a unique and invaluable opportunity to grow as an academic. Dr Mandato – thank you for trusting me with ANAT 261 all these years. It showed me how much I love teaching. Dr Greenwood – thank you for your patience and teachings, I will always remember our weekly lab meetings at the train station.

I would also like to thank Dr Boucher for his endless years of support and mentoring of me. Eric you have been invaluable to my success, I do not know what I would have done with out you. Thank you for your teachings, jokes and energy.

In addition, I would like to acknowledge the members of my PhD committee, Dr Bui, Dr Tanny, and Dr Ribeiro-da-Silva, for their time and valuable advice.

To all the members of Mandato and Greenwood labs, thank you for your help and friendship. You have all made a lasting impact on my graduate experience.

Lastly, thank you to my family. Thank you for your love and support (and money). I hope I made you proud.

Contribution of Authors

Greenwood, M.T

Principle investigator for all projects included in this thesis.

Mandato, C.A

Co-supervisor for all projects included in this thesis.

Zhou, D.R

Performed all experiments, imaging, statistical analyses for all projects in included in this thesis and the writing of this thesis.

Boucher, E

Mentor and lab manager. Assisted and advised on most experiments included in this thesis.

Original Works Included In Thesis

Published

Eid R, **Zhou DR**, Arab NTT, Boucher E, Young PG, Mandato CA, Greenwood MT. *Heterologous expression of anti-apoptotic human 14-3-3β/α enhances iron-mediated programmed cell death in yeast.* PLoS One. 2017 Aug 30;12(8):e0184151.

In this study, we characterize the response of yeast heterologously expressing pro-survival sequences to different RCD-inducing stresses including excess copper and iron. We show here that iron-mediated RCD in yeast is distinct from copper in that it can be enhanced by the expression of pro-survival sequences and requires a functional vacuole.

Zhou, D. R., Eid, R., Miller, K. A., Boucher, E., Mandato, C. A., & Greenwood, M. T. (2019). *Intracellular second messengers mediate stress inducible hormesis and Programmed Cell Death: A review*. Biochimica et biophysica acta. Molecular cell research, 1866(5), 773–792.

In this review, we discuss how cells respond to stress by increasing the levels of a diverse group of intracellular second messengers including both familiar (e.g. ROS) and less well known (e.g. iron) molecules.

Zhou, D. R., Eid, R., Boucher, E., Miller, K. A., Mandato, C. A., & Greenwood, M. T. (2019*). Stress is an agonist for the induction of programmed cell death: A review.* Biochimica et biophysica acta. Molecular cell research, 1866(4), 699–712

In this review, we re-examine the relationship between stress and cellular response. We focus on several other, often overlooked observations of stress responses such as the concept of the intracellular milieu which suggests that cells actively perform homeostasis in part by limiting the entry to most extracellular chemicals. In particular, we focus on the concept that stress is an agonist that is transduced into the cell to activated regulated cell death or survival responses.

Zhou, D. R., Miller, K. A., Greenwood, M., Boucher, E., Mandato, C. A., & Greenwood, M. T. (2020*). Correcting an instance of synthetic lethality with a pro-survival sequence.* Biochimica et biophysica acta. Molecular cell research, 1867(9), 118734.

In this study, we report on the characterization of human LIM domain containing CSRP3 as a novel suppressor of Bax and a pro-survival sequence when heterologously expressed in yeast. Additionally, we are the first to systematically examine the role of LIM domains in pro-survival responses.

In Preparation

Zhou, D. R., Miller, K. A., Eid, R., Scott, J., Lebel, O., Mandatom C. A., & Greenwood, M. T. (2022). *Iron insolubility limits the toxicity of excess extracellular iron.*

In this study, we examine the toxic effects of excess iron in cell culture media and show that the accumulation of a precipitate forms in a dose dependent manner that precedes the RCD-inducing effects of iron.

List of Abbreviations

Accidental Cell Death (ACD)

Bcl-2 associated X protein (Bax)

B-cell lymphoma 2 (Bcl2)

Cysteine and glycine rich protein 3 (CSRP3)

Regulated cell death (RCD)

Regulated cell survival (RCS)

Reactive oxygen species (ROS)

Mitochondrial outer membrane permeabilization (MOMP)

Yeast nitrogen base (YNB)

Yeast nitrogen base with galactose and raffinose (YNBG/R)

Yeast nitrogen base with glucose (YNBD)

Chapter 1.

Introduction & Literature Review

1.2. Cellular Homeostasis and Stress

"A crust eaten in peace is better than a banquet partaken in anxiety"

-Aesop

There exists an important relationship between a cell and its environment that directly influences whether it will live or die. On one hand, a cell's internal environment is intrinsically different from its extracellular environment. By many criteria, this is the definition of a cell as it actively maintains what Claude Bernard termed the intracellular milieu. On the other hand, the cell is not completely independent of its surroundings. For life to exist, this external environment must also contain and allow access to appropriate macro and micro-nutrients, amenable physical-chemical conditions as well as being relatively free of stressors. Thus, it reasonably follows that any perturbations to these parameters poses a risk to cellular viability.

Importantly, the cell is not completely at the mercy of its environment. A key function in support of life is the ability to perform homeostasis. Homeostasis refers to a system of self-regulating processes that aim to maintain stable internal conditions in response to external changes or stress. While the term stress is difficult to define precisely, a basic working definition is that it is anything that may be perceived as having or indeed have the potential to disrupt to homeostasis (Zhou, Eid, Boucher, et al., 2019). Thus, cellular homeostasis is achieved through the regulation of various genetically encoded processes that aim to resist stress and maintain the intracellular milieu. These homeostatic processes are both numerous, complex and with limits. In fact, the relationship between cellular stress and cellular response to stress can be modeled as a graded response in which the type of response is directly proportional to the intensity of stress (Figure 1)

(Agathokleous & Calabrese, 2019; Aki et al., 2015; Galluzzi et al., 2018; Zhou, Eid, Boucher, et al., 2019). In the face of stress, cells can choose either to adapt to promote its own survival by activating protective hormetic processes, initiate genetically programmed cell death processes, or under extreme conditions consequentially die as is typical in necrosis. Thus, the relationship between cellular stress and stress response is in many ways a description of the how cells live in nature.

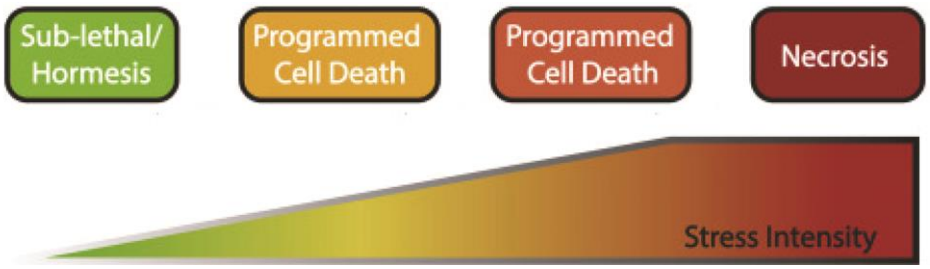

Figure 1. Stress response is a graded one that is dictated by stress intensity*. The intensity of stress is directly related to the kind of response cells may assume. At low levels of stress, cells may either adapt or activate protective hermetic processes to maintain homeostasis. As the intensity of stress increases, cells may instead choose to initiate genetically encoded cell death processes (RCD) and actively die. At stress levels far beyond what is necessary to induce RCD, cells die independent of regulation as is typical in necrosis. (Zhou, Eid, Boucher, et al., 2019)*

1.2.1. Extracellular Stresses

The conditions of the extracellular milieu often influence whether a cell will live or die. Indeed, most of the forms of stress cells encounter originate from their external environment. Here, some common external stresses are discussed.

1.2.1.1. Temperature

Temperature has a direct and significant impact on the viability of both life and the molecules necessary for life. Life on earth has adapted to a plethora of climate conditions but seems nonetheless limited to temperatures approximately between -15C and 115C (McKay, 2014). The reason for this mostly relates to the effects of temperature on key biochemical processes and molecules. At low temperatures, the rate at which biochemical reactions necessary for life may proceed too slowly as molecules are insufficiently imbued with kinetic energy. At too high temperatures, key biological molecules such as lipid bilayers, proteins and DNA may breakdown or denature. Importantly, among these molecules are enzymes. Enzymes play key roles in biology as they catalyze essential biochemical processes in cells. In addition to its effects on the physical integrity of molecules, temperature also has a direct impact on enzyme activity and kinetics (Peterson et al., 2007). Collisions between molecules increases with temperature and lends to increase reaction rates between enzymes and their substrates. However, as temperature continues to increase, enzymes experience the same fate as other proteins and risk becoming denatured, reducing reaction rate.

Experimentally, the effect of temperature on cell viability and growth is readily observable in in all organisms including both yeast and bacterial cell cultures. The eukaryotic yeast *Saccharomyces cerevisiae* can be grown in culture at room temperature but grow optimally at 30C (Bullerman, 2003). This is accompanied by near-100% cell viability in laboratory cultures ((Eid, Zhou, et al., 2017; Zhou et al., 2020)). As culturing temperature increases, the cell can induce hormetic processes and survive at elevated temperatures. For example, the mild stress of growing at 37C has been found to increase the chronological lifespan of yeast in culture (Baldi et al., 2017).

Further increases will eventually lead to the loss of cell viability. Indeed, cell viability in cultured yeast has been shown to be impossible above 45C (Munna et al., 2015). In *E. coli*, growth is optimal at 37C but heat shocks at 42C can induces protective effects that limit cell death, but cell viability decreases as temperature increases (Nabiilah, 2021; Russell, 2003).

In response to temperature stress, cells typically respond by upregulating the expression of heat stress-responsive genes. The heat shock protein (HSP) family is conserved in virtually all living organisms and encode several members that largely serve chaperone functions, ensuring the proper folding and integrity of proteins and other cellular macromolecules under stress (Matz et al., 1995). Interestingly, HSP function and expression has also been reported in response to other stresses such as cold conditions and pro-apoptotic signalling mechanisms suggesting a more general protective role (Beere, 2004; Chinnusamy et al., 2010). In fact, many tumor cells are known to express atypical levels of HSP genes (Jolly & Morimoto, 2000). In studies of yeast lacking HSP orthologues, cells demonstrate growth restriction as well as decreased cell viability in response to increasing temperatures that are otherwise tolerable in control cells (Kimura et al., 1994; Lindquist & Kim, 1996).

Importantly, the use of temperature stress as a growth restricting and cell death-inducing agent has also been a fundamental strategy for the identification of essential genetic networks. Hartwell's Nobel prize winning studies in the late 1960's with temperature sensitive *S. cerevisiae* strains resulted in the identification of over 400 temperature sensitive mutations involved in a range of essential processes including cell division, protein, DNA and RNA synthesis (Hartwell, 1967). Such findings have been invaluable to the elucidation the function of genes, their protein products as well as identifying of essential gene networks.

1.2.1.2. Osmotic Stress

The biological processes and structures that exist within a cell are in many ways directly related to the volume and spatial organization of the cell itself (Delpire & Gagnon, 2018). These include cellular concentrations of proteins and other molecules as well as the organisation of physical molecular structures such as the cytoskeleton (Hohmann, 2009). Thus, changes to cellular volume and shape have the potential to disrupt homeostasis. To protect against this, cells strictly regulate the movement of material between the intra- and extracellular space across the plasma membrane. In particular, the movement water (which comprise ~ 70% of a single cell's volume) is tightly regulated (Strange, 2004; Yasui, 2014). Indeed, cells possess specialized membrane channels such as aquaporins that direct the flow of water (Rump & Adamzik, 2018). However, the membrane is not entirely impermeable to water, making the cell subject to osmotic forces.

A consequence of this permeability is the need to contend with differences in osmolarity between intra- and extracellular environments. In hypo-osmotic environments, osmotic forces direct the flow of water into cells causing swelling and potentially the rupture of the cell membrane and lysis of the cell. In the face of dynamic external osmolarity, cells may respond by regulating the expression of aquaporins to stabilise the flux of water (Booth & Louis, 1999; Koike et al., 2021). Cells in multicellular organisms tend to avoid this problem by having their internal extracellular fluids regulated by central processes (Delpire & Gagnon, 2018). Single cells organisms that must directly content with changing environmental conditions like the eukaryotic yeast have further solved this problem by evolving a cell wall. The cell wall provides additional protection against hypo-osmotic shock by serving as a physical barrier limiting the expansion of the plasma membrane against it (Hohmann, 2002). In hyper-osmotic environments, outward flux

of water leads to water loss and cell shrinkage; potentially inducing cell death (Stohr & Rehm, 2021). One protective mechanism against hyper-osmotic stress is the upregulation of de novo synthesis of glycerol via the high osmolarity glycerol (HOG) pathway and the downregulation of aquaporins as well as glycerol channels (Hohmann, 2002; Mager & Siderius, 2002). The stimulation of the HOG pathway in yeast leads to osmoadaptation in two ways. At the membrane, expression of Hog1p or HOG1 inhibits the outward flux and promotes the inward transport of glycerol via inhibition and activation of Fps1p and Stl1p respectively (de Nadal & Posas, 2022). In yeast that express mutant Fps1p that are constitutively do not accumulate intracellular glycerol and struggle to grow in hyperosmotic environments (Hohmann et al., 2007). Increasing intracellular glycerol serves to raise intracellular osmolarity to return to isotonic conditions. In addition to the regulation of glycerol flux, Hog1 promotes the expression of genes encoding endogenous glycerol producing elements such as GPD1 and GPP2 (Babazadeh et al., 2014). Thus, it follows that in experiments with HOG signalling mutants, compared to control, cells under hyperosmotic stress show increased osmosensitivity as evidenced by decreased growth and viability (Prick et al., 2006).

1.2.1.3. Exogenous Metals Salts

Heavy metals are naturally occurring elements that aid in the function of several key biological processes such as oxygen binding in heme proteins, the electron transport chain, and the conduction of electrical impulses in nervous tissue. These metals include but are not limited to sodium, potassium, magnesium, calcium, copper, and iron (Tchounwou et al., 2012). Importantly, these metals exist at very low concentrations in biological systems. Indeed, heavy metals have a

myriad of other applications within industry, agriculture, medicine, and technology which raises concerns over their effect on both the environment and human health. For example, the most common heavy metals in wastewater include arsenic, cadmium, chromium, lead, nickel, zinc, and copper; all of which are known to pose risks to human health at high levels (Lambert M, 2000). In fact, arsenic, cadmium, chromium and nickel are classified as group 1 carcinogens by the International Agency for Research on Cancer.

For many, the exact mechanism by which the presence of excess exogenous metal salts is transduced to a cell death-inducing stress is not fully understood. Most models of metal toxicity posit that these stresses enter the cell and promote the production of reactive oxygen species (ROS; reviewed later) leading to irreversible cell damage and death (Balali-Mood et al., 2021). Nonetheless, there exists a continuous demand for metal toxicity studies in both mammalian cells and yeast.

1.2.1.3.1. Copper

Ionic copper is a trace metal element that is found in the active sites of a myriad of enzymes, making it an essential mineral for life. Under normal physiological conditions, copper can enter cells via the transmembrane transporter 1 (Crt1) and divalent metal transporter 1 (Dmt1) (Nose et al., 2006). In cells such as enterocytes, copper is captured and transported by the chaperone antioxidant protein 1 (Atox1) to a copper ATPase pump (ATP7A/B) for secretion (Lutsenko et al., 2007). Simultaneously, other transport processes distribute copper as needed to areas that require it (Banci et al., 2010). In large, free ionic copper is virtually non-existent in the cytosol (Kaplan & Maryon, 2016).

Copper is of clinical interest as its intracellular accumulation is seen in human disease. Wilson's disease is an inherited disease caused by a mutation in ATPB7 which impairs copper efflux in hepatic cells (Chang & Hahn, 2017). The resulting accumulation of intracellular copper subsequently leads to cell death and organ failure (Mayo Clinic, 2022). Interestingly, the precise mechanism by which excess extracellular copper leads to cell death remains obscure. Like other forms of ionic metal stress, copper cytotoxicity is most commonly linked to increased levels of intracellular reactive oxygen species (ROS) (Eid et al., 2016; Husain & Mahmood, 2019; Zhou et al., 2020). As a transition metal with multiple oxidation states, copper is thought to propagate and generate free radical species which can potentially lead to intracellular damage (Zhou, Eid, Miller, et al., 2019). Indeed, the generation and accumulation of intracellular ROS is a common marker of stress and a fundamental tenet of the oxidative stress model (reviewed later).

To understand copper toxicity, several models have been examined. In yeast, the addition of copper to growing cultures induces regulated cell death (Eid et al., 2016; Eid, Zhou, et al., 2017; Zhou et al., 2020{Eid, 2016 #74). The cell death inducing effects of copper are also accompanied by detectable increases in reactive oxygen species; a common precursor marker of cell death (Eid et al., 2016; Eid, Zhou, et al., 2017; Zhou et al., 2020). Of importance is that copper-mediated cell death can be prevented by the ectopic expression of pro-survival sequences (Eid et al., 2016; Eid, Zhou, et al., 2017; Zhou et al., 2020). This indicates that copper does indeed lead to a form of RCD (Galluzzi et al., 2018). In mammalian cell cultures, excess exogenous copper has also been shown to readily induce apoptosis (Agarwal et al., 1989). Thus, copper is an effective, clinically relevant cell death inducing agent and the mechanisms of copper toxicity and RCD induction is an important area of investigation.

In effect, a recent study published in *Science* reported on a large study aimed at defining the role of copper in cell death (Tsvetkov et al., 2022). Using cultured mammalian cells, it was found that excess copper leads to toxicity by targeting lipoylated TCA cycle proteins. A clear limitation of the study was that a copper ionophore was required to study the effects of copper toxicity. This is required, as the study admits because the cell is largely impermeable to excess copper. Our own studies also indicate that copper-mediated RCD requires a ~2000-fold excess of copper relative to what exists in normal cell culture media (Eid et al., 2016; Eid, Zhou, et al., 2017; Zhou et al., 2020). As we reviewed recently, homeostatic mechanisms prevent excess nutrients, even micronutrients like copper from entering cells. While the exact mechanism in which copper induces cell death is unknown, possible mechanisms likely involves cell surface transporters and receptors and transduction to intracellular second messenger systems (Zhou, Eid, Miller, et al., 2019).

1.2.1.3.1. Iron

Iron is trace element that is an essential micronutrient required for numerous biological processes (Ems et al., 2022). Most notably, it plays a key role in the transport of oxygen in blood as it is an essential component of heme proteins. In our bodies, biologically available iron is so rare that once captured, it is not removed. Iron is tightly hoarded by our bodies and stored in what appears to be dedicated vesicles that are present in the cytoplasms of cardiac, skeletal, and liver cells (Saito, 2014). This can be problematic in certain disease states like hemochromatosis; a disease characterized by excess iron uptake leading to high iron levels and failure of multiple organs. These conditions can arise due to genetic mutations that lead to defective iron metabolism

but can also be the result of chronic oral consumption as is common in patients with anemia (Lands & Isang, 2017). Thus, like copper, iron is a commonly studied cellular stress.

Under physiological conditions, iron in circulation is bound by the glycoprotein transferrin (Saito, 2014). When bound, iron is absorbed into cells by binding a cell surface transferrin receptor and subsequently incorporated into an endosome (Octave et al., 1983). Inside the cell, iron is then transferred to ferritin where it is stored in specialized structures (Lane et al., 2015; Octave et al., 1983). Thus, it appears that while iron is absorbed by cells, it is ultimately compartmentalized and removed from labile circulation.

As an ion, iron exists in two forms, iron (II) and iron (III). The ability to switch between oxidative states allows iron to generate and react with reactive oxygen species through Fenton Reactions (Bystrom et al., 2014). Similarly, exogenous iron stress is linked to enhanced intracellular levels of ROS, oxidative stress, and subsequent cell death (Eaton & Qian, 2002; Eid, Zhou, et al., 2017) . In yeast, studies with exogenously supplied iron have shown that in excess, it can inhibit growth and induce cell death (Eid, Zhou, et al., 2017). While the oxidative stress model provides a possible explanation for iron-mediated cell death, the exact mechanism by which iron induces death remains unclear. In some studies, the use of intracellular iron chelators in the presence of iron stress has been shown by some to protect against its deleterious effects (Hatcher et al., 2009). Interestingly, these same chelators in some cases are also effective at protecting against other stresses unrelated to iron; suggesting that cell death due to excess iron may be the result of a more general regulated pro-death process (Reeder et al., 2008). In our studies with iron, the response of yeast seems to differ from copper in that the expression of pro-survival sequences do not protect against it but rather enhance its deleterious effects (Eid, Zhou, et al., 2017). This

contrasting response is observable with multiple pro-survival sequences and suggests the existence of a distinct iron-mediated RCD pathway (Eid, Zhou, et al., 2017).

1.2.1.3.1. Lithium

Lithium is not an essential mineral but is routinely prescribed as a therapy for bipolar disorder and other neuropsychoses (Curran & Ravindran, 2014; Kilts, 1998; Volkmann et al., 2020). Despite its extensive usage and clinical success, its precise mechanism of action in cells is also not known. Furthermore, lithium therapy is complicated by the fact that the range of concentration required to be therapeutic is narrow. Indeed, many patients on chronic lithium experience at least one episode of lithium toxicity (Amdisen, 1988). As such, lithium toxicity is of interest for many. A widely believed mechanisms of action for lithium action is the depletion of inositol by inhibition of inositol monophosphatases (IMPase) leading to the breakdown of the inositol cycle and calcium signalling (Lopez et al., 1999; Vaden et al., 2001). To clarify this issue, yeast have been used as model organisms for both lithium action and toxicity. Thus, excess lithium was also found to result in decreased intracellular mass of inositol while simultaneously upregulating expression of *de novo* inositol biosynthesis regulators INO1 and INO2 (Vaden et al., 2001). Interestingly, in other studies, the combined null mutations in all two yeast IMPases had no deleterious effect on growth or lithium tolerance suggesting that the inositol depletion hypothesis is insufficient to explain the effects of lithium in yeast (Lopez et al., 1999).

Other possible targets directing lithium effects have been studied. In addition to the regulation of inositol, lithium is also believed to be an inhibitor of glycogen synthase kinase 3 (GSK3) which regulates and targets a diverse array of processes in cells (Gould et al., 2004;

O'Brien & Klein, 2009). Given its involvement in many cellular processes, the loss of GSK3 inhibition by lithium may serve to promote downstream pathways. On the other hand, studies using yeast have shown that excess lithium can induce both cell death and growth inhibition (Dichtl et al., 1997; Martins et al., 2008). In this thesis, our studies show that the toxic effects of lithium can be protected against by the ectopic expression of pro-survival sequences. Thus, the involvement of RCD is a possible effect of excess lithium.

1.2.2. Oxidative Stress and Reactive Oxygen Species

Many chemical reactions within a cell require the movement of electrons. This is commonly observed in the mitochondria where the movement of electrons drives the generation of cellular energy (Zhao et al., 2019). These and other processes generate a great number of free radicals that are potentially toxic and cell death inducing (El-Osta & Circu, 2016). The term free radical refers to molecules with unpaired electrons in their outer shells making them highly reactive in this manner. These commonly exist in all cells as they appear as intermediates in redox reactions. In particular, reactive oxygen species (ROS) are likely the most frequently encountered species in such reactions. As an electron acceptor, molecular oxygen participates in several key reactions such as the interconversion between water and hydrogen peroxide (; below). Of note, is the formation of the intermediate free radical species superoxide ($O_2^{\bullet -}$), peroxide ($O_2^{\bullet 2-}$) as well as hydroxide ($OH^{\bullet}$).

$$H_2O + é \rightarrow O_2^{\bullet -} + é \rightarrow O_2^{2-} + 2H^+ \rightarrow H_2O_2 + é \rightarrow OH^{\bullet} + OH^- \rightarrow H_2O$$

Due to their reactive nature, these species can react with organic substrates such as lipids, proteins and DNA. In the absence of regulation, these free electron species can also damage cellular macromolecules and organelles giving rise to deleterious effects for the cell (Pham-Huy et al., 2008; Zhou, Eid, Boucher, et al., 2019; Zhou, Eid, Miller, et al., 2019). Unsurprisingly, cells have evolved several ways to capture free electrons using molecules like NAD+, GSH and TSH as well as enzymes including catalase and peroxidase (Zhou, Eid, Boucher, et al., 2019; Zhou, Eid, Miller, et al., 2019). Thus, in short, under normal conditions, cells maintain a steady state redox balance between reactive species-producing and removal systems.

Oxidative stress is a phenomenon derived from an imbalance within either of these processes that results in the accumulation of reactive species. The accumulation of intracellular ROS in response to stress is a commonly observed response to virtually all stress (Ho et al., 2013). Indeed, the accumulation of ROS is the proposed mechanism of action for many forms of stress such as exogenous metal salts whereby cytotoxic ions with multiple oxidation states or material enters the cell and interact with ROS generating mechanisms (Zhou, Eid, Boucher, et al., 2019; Zhou, Eid, Miller, et al., 2019). This then leads to increased intracellular levels of ROS which consequentially lead to irreparable damage of cellular macromolecules and structures resulting in cell death. Thus, the detection of elevated intracellular ROS is a widely accepted marker of stress and to some extent, an indicator of the induction of regulated cell death.

While cellular stress is closely linked to increases in intracellular ROS, the precise mechanism by which stress is transduced to ROS is not fully agreed upon. Despite this, many mechanisms are proposed and may explain how stress manifests itself as an increase in intracellular ROS. As previously discussed, a common model of metal toxicity is the entry of stress into cells and direct interaction with ROS generating processes. These models however conflict

with homeostatic principles. Indeed, studies that have examined the change in intracellular iron after exposure to excess exogenous iron only observed modest increases in intracellular iron (Jacobs et al., 1978; Zombola et al., 1979). Elsewhere, metal stressors have also been shown to affect mitochondrial electron transport potentially by interfering with iron-containing sulfur clusters naturally required for normal electron movement thus leading to electron leakage (Rose et al., 2017). During periods of starvation or energy depletion, increases in ROS or oxidative stress may be explained by a decrease in ATP:ADP and NADH: NAD+ ratios (Ursini et al., 2016).

Despite this, the oxidative stress model is ultimately complicated by the fact that ROS is necessary but not sufficient to induce regulated cell death (Zhou, Eid, Boucher, et al., 2019). There are many experiments that have sought to reduce intracellular ROS but do not necessarily observe a reduction in cell death (Ghezzi et al., 2017; Zhou, Eid, Boucher, et al., 2019). Nonetheless, oxidative stress remains both a highly researched but also highly marketed model for the way stress relates to cellular viability and clinical health. Elevated ROS simply represents one way in which we understand it.

1.3. Cellular Responses to Stress

1.3.1. Stress Intensity Dictates Cellular Response to Stress

Given the myriad of different stresses that a cell may face, it is also equipped with several responses. The adage "the dose is the poison" is aptly suited to describe how cells relate stress to their own responses. Indeed, it is the intensity of a stress that most relates to the cellular response. As previously mentioned, the cellular stress response is a graded one that depends on the magnitude of stress (Figure 1). Here, several of these responses will be discussed.

1.3.2. Sublethal Stress: Pre-Conditioning & Hormesis

The concept of pre-conditioning and hormesis are based on the fact stress can present at levels that have the potential to disrupt homeostasis but not enough to overpower normal homeostatic mechanisms (Agathokleous & Calabrese, 2019; Zhou, Eid, Miller, et al., 2019). Within this range of stress intensity, the cellular response can have both adaptive and cyto-protective effects. On one hand, in response to mild stress, cells may simply adapt by performing physiological homeostasis to return to normal conditions. For example, in the presence of excess metal salts or in the absence of nutrients, cells may regulate the activity and number of cell surface transporters to adjust the rate of absorption into the cell (Bashir et al., 2016; Bleackley & Macgillivray, 2011; Chandrangsu et al., 2017; Recalcati et al., 2010). As mentioned above, this type of regulation is also seen with the regulation of glycerol flux across the cellular membrane in hyperosmotic shock experiments (Hohmann, 2002; Mager & Siderius, 2002; Rump & Adamzik,

2018). On the other hand, mild, sub-lethal stress can also stimulate cyto-protective processes (see 1.3.4). For example, in response to mild increases in temperature, cells typically upregulate the expression of heat responsive genes that serve to ensure proper protein folding and chaperone functions (Baldi et al., 2017). In *C. elegans*, pre-treatment with temperature, UV and gamma radiation was reported to induce tolerance to phosphine toxicity (Alzahrani & Ebert, 2018). In industrial applications of yeast, pre-treating yeast with sodium chloride is a commonly used strategy to increase fermentation and ethanol yield (Logothetis et al., 2010). In either situation, the low-dose stimulation by certain stresses serves to active adaptive cyto-protective processes that can increase tolerance to subsequent stresses. This phenomenon is commonly referred to as pre-conditioning.

1.3.3. Cell Death

While death is the inevitable fate for all cells, it is not a ubiquitously passive consequence of life. Indeed, there exists many active processes that cells can deliberately activate to manifest their own demise. In response to high levels of stress, cells may die due to the induction of regulated death processes or die via unregulated, catastrophic or necrotic cell death. However, it is firstly important to understand the distinction between what is regulated and non-regulated cell death as the two are related, but fundamentally different in their meaning and importance.

1.3.3.1. Regulated Cell Death

In the early 19th century, developmental biologists were plagued by the mystery of how certain structures in developing embryos regressed or entirely disappeared. Once Schleiden and Schwann established the cell theory in 1842, reports of cell death came soon after that same year as developmental biologists like Carl Vogt noted the disappearance of cells in the notochord of metamorphic toads as they were replaced by the forming vertebrae (Clarke & Clarke, 1996; Zakeri & Lockshin, 2008). Over the next century, scientists observed dying cells not just during development, but in other non-metamorphic tissues and organisms, ultimately establishing cell death as a biological activity. Today we understand regulated cell death as both a common and necessary phenomenon required for the proper development and maintenance of tissues, organisms, and cellular populations. Indeed, cell death is not a ubiquitously random consequence of life, but often a calculated, deliberate and genetically coded action.

Regulated cell death (RCD) refers to the collection of genetically encoded processes that are activated in response to stress that guide cells towards a controlled and organized death. According to the cell death nomenclature committee, RCD is defined by the following tenets (Galluzzi et al., 2018):

1. RCD is a genetically encoded process(es).
2. RCD can be modulated by genetic or pharmacological intervention.

Importantly, the second tenet is experimentally very useful. Thus, we and others make use of the fact that the prevention of cell death in response to stress by the overexpression of pro-survival sequences is indicative that RCD is observed (Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012; Eid et al., 2014; Eid, Zhou, et al., 2017; Horowitz et al., 2013; Jones et al., 2015; Sheibani et al., 2015; Zhou et al., 2020).

RCD plays several extremely important roles in biology. During development, RCD serves to sculpt tissues and organs to their final morphology (Renehan et al., 2001). For example, RCD is the mechanism responsible for the separation of digits as they develop and the pruning of synapses between neurons in the developing brain (Geden et al., 2019; Gilbert, 2000). RCD also serves homeostatic functions such as in the housekeeping turnover of internal and external epithelia (von Herbay & Rudi, 2000). Thus, it is not surprising that dysregulated RCD is associated with most, if not all disease states. These include the obvious diseases characterized by pathological or abnormal cell death (i.e. neurodegenerative (Parkinson's) and autoimmune diseases (Type 1 diabetes)) or pathological survival of cells (i.e. cancer) as well as less obvious states such as infections (i.e. viral) that can induce RCD (Thomson, 2001). In this regard, the study of RCD remains important as we strive to understand how cells choose to live and die.

1.3.3.1.1. Apoptosis

Under the umbrella of RCD, several forms of genetically controlled cell death processes and routines have been identified. By far, the most studied and understood form of RCD is apoptosis (Singh et al., 2019). The term apoptosis mostly refers to a set of morphological features that accompanies this type of cell death. This includes, membrane blebbing, condensation of nuclear chromatin, disassembly of intracellular both organelles and structures and finally, the breakdown of the cell itself into smaller fragments known as apoptotic bodies (S. Elmore, 2007). Apoptosis is typically measured and defined using these morphological markers but is also routinely assessed using molecular markers. Flow cytometric assays can take advantage of molecular changes to the cell membrane itself and its integrity during apoptosis.

Phosphatidylserine is a phospholipid that comprises one of many different plasma membrane components. Under normal non-apoptotic conditions, it is positioned on the inner leaflet of the plasma membrane. During apoptosis, certain membrane proteins such as flippase transfer phosphatidylserine to the outer leaflet exposing it to the cell surface (Nagata et al., 2016). There, phosphatidyl serine plays a key role in marking cells for apoptotic clearance by serving as "eat me" signals to nearby phagocytes (S. Elmore, 2007). Experimentally, externalized phosphatidyl serine can be detected using binding assays with molecules such as annexin V. In addition to cell surface markers of apoptosis, it can be further measured by examining the physical integrity of the plasma membrane. Vital dyes such as propidium iodide and trypan blue operate on the evidence that viable or non-apoptotic cells have intact plasma membranes that are relatively impermeable to polar molecules. Thus, the use of such dyes are useful and allow for visual discrimination between all live and dead cells (S. Elmore, 2007).

Molecularly, apoptosis is manifested largely by the activity of caspases (S. Elmore, 2007; Li & Yuan, 2008). During apoptosis, cells shrink, cytoskeleton dissembles, and the nuclear envelope disappears in preparation for the formation of smaller self-enclosed apoptotic bodies. The caspase family proteases are primarily responsible for driving these morphological changes as they directly mediate the breakdown and degradation of internal cellular proteins thus leading to the breakdown of cellular components such as the cytoskeleton and organelles. Under normal non-apoptotic conditions, caspases are synthesized as inactive zymogens or pro-caspases which are cleaved when activated. The apoptotic caspase signalling cascade involves two groups of caspases – initiator and executioner caspases. Initiator caspases include caspase 2, 3, 8, 9, and 10 while executioner caspases include caspase 3, 6, and 7. It is ultimately the action of the executioner caspases that bring the physical disassembly of the cell and resulting morphological changes. Thus

experimentally, the detection of active executioner caspases such as caspase 3 often serves as markers for the induction apoptosis (S. Elmore, 2007).

The processes that initiate apoptosis upstream of caspase activation are both numerous and complex. In general, they are two distinct processes that share crosstalk with each other, and are commonly referred to as the intrinsic and extrinsic pathways. Studies of these pathways continue to inform our understanding not only about apoptosis but also RCD.

1.3.3.1.2. Intrinsic Pathway

The intrinsic pathway or mitochondrial pathway refers to apoptotic processes and events that center around the mitochondria during stress. The functional consequence of pro-apoptotic signalling through the intrinsic pathway is the disruption of the mitochondrial membrane in an event called mitochondrial outer membrane permeabilization (MOMP), resulting in the release of cytochrome-c into the cytoplasm (S. Elmore, 2007; Fulda & Debatin, 2006; Li & Yuan, 2008). Under normal conditions, cytochrome c is embedded in the mitochondrial membrane as an electron transporter. During apoptosis, cytochrome c complexes with apoptotic protease activating factor 1 (APAF1) to form a pro-apoptotic complex in the cytosol called the apoptosome. This complex then serves to couple with ATP to hydrolyze and activate caspase 9 zymogens which in turn activate the executioner caspases 3, 6 and 7 (S. Elmore, 2007).

In addition, to cytochrome c, MOMP also causes the release of other pro-apoptotic molecules including Smac/DIABLO and endonuclease G into the cytosol (S. Elmore, 2007). In the cytosol, Smac/DIABLO indirectly promotes apoptosis by inhibiting the anti-apoptotic inhibitor of apoptosis (IAP) protein family members. IAPs function primarily to inhibit caspase activity.

Once released, endonuclease G participates as a nuclease mediating DNA breakdown once it translocates from the mitochondria to the nucleus.

Intrinsic apoptotic signalling is tightly regulated by the B-cell lymphoma 2 (Bcl-2) protein family around the outer mitochondrial membrane (Kale et al., 2018; Tsujimoto, 1998). Bcl-2 family members interestingly possess both pro and anti-apoptotic functions but are structurally related by the presence of Bcl-2 homolgy 3 (BH3) domains . The anti-apoptotic members Bcl-2, Bcl-2L and Bcl-XL prevent cyt-c release while pro-apoptotic members residing in the cytosol such as BAX translocate to the mitochondria and promote MOMP either by binding the mitochondria directly as dimers and forming pores or by binding anti-apoptotic Bcl2 family members via their BH3 domains and antagonizing their activity (Singh et al., 2019).

Apoptotic signalling via the intrinsic pathway is usually in response to internal injury or external stress. Interestingly, the expression of mammalian BAX in yeast also leads to a RCD that resembles apoptotic cell death in mammalian cells (Khoury & Greenwood, 2008; Ligr et al., 1998). In this regard, BAX remains an attractive apoptotic regulator for study as its role in mediating MOMP lies at the center of many RCD contexts.In effect, many therapeutic strategies are currently being developed against Bax with the expectation that they serve to decrease enhanced RCD in certain pathologies like Parkinson's disease (Bernardini et al., 2019; Erekat, 2018).

Unlike extrinsic or receptor mediated apoptotic signalling, the precise mechanism in which stress is transduced to the mitochondria leading to MOMP is not fully understood.

To this end, yeast have been used extensively as model organisms to study and model stress-mediated RCD (Clapp, Portt, Khoury, Sheibani, Eid, et al., 2012). It is likely that stress itself acts as an agonist for regulated stress responses and is transduced by multiple second messenger systems including ROS (Zhou, Eid, Boucher, et al., 2019; Zhou, Eid, Miller, et al., 2019). It is of

interest that many pro-survival sequences discussed in this supress the effects of Bax as well as prevent RCD in response to other stresses like copper.

1.3.3.1.3. Extrinsic Pathway

The extrinsic pathway or receptor mediated pathway refers to pro-apoptotic signalling mechanisms that are the result of the binding of death ligands to cell surface death receptors (Clapp, Portt, Khoury, Sheibani, Eid, et al., 2012; Singh et al., 2019). Such examples include tumor necrosis factor (TNF) and its receptor TNFR1 as well as Fas receptor and Fas ligand. When bound by their respective death ligands (TNF-a and Fas ligand respectively) they adapter molecules including TRADD and FADD to the inner membrane. Here, this complex interacts with procaspase 8. The activated caspase 8 then can either directly initiate the executioner caspase cascade ending with caspase 3 or it can interact with pro-apoptotic Bcl-2 family member proteins including BID and BAX to initiate MOMP and initiate the intrinsic pathway (Susan Elmore, 2007; Fulda & Debatin, 2006). Thus, the extrinsic pathway shares crosstalk with the intrinsic pathway while simultaneously capable of initiating the caspase signalling cascade that ultimately ends with caspase 3.

1.3.3.1.4. Necroptosis

The stimulation of the extrinsic pathway through death receptors can also result in an alternate cell death modality termed necroptosis. Necroptosis was first elucidated in experiments that stimulated extrinsic apoptotic pathway signalling but inhibited caspase 8 resulting in a cell

death that morphologically resembled necrosis (Dhuriya & Sharma, 2018; Vanden Berghe et al., 2014). However, like apoptosis, necroptosis is genetically regulated. The recruitment of TRADD to the membrane by TNFR can alternatively complex with the necroptotic kinases RIPK1/3 to form what is called the necroptosome. The necroptosome then activates mixed lineage kinase (MLKL) which when cleaved, translocates to the inner leaflet of the plasma membrane where it compromises its integrity by binding phosphatidylinositol phosphates (Dhuriya & Sharma, 2018; Vanden Berghe et al., 2014).

The regulation and activation of necroptosis is believed to be dependent on two factors: 1) the inhibition of caspase 8 and 2) the activation of RIPK and MLKL (Vanden Berghe et al., 2014). As a form of RCD (rather than necrosis), necroptosis has also been shown to be subject to inhibition by molecules including necrostatin 1 which directly inhibitors RIPK1 (Degterev et al., 2005). Interestingly, the activation of RIPK3 from RIPK1 is also antagonized by pro-apoptotic proteins including caspase 6, 8 and 10 suggesting that necroptosis shares cross talk with apoptosis and may arise under conditions where apoptosis is not possible (Green et al., 2011; Huang et al., 2021; Someda et al., 2020).

1.3.3.2. Necrosis/Accidental Cell Death

Non-regulated cell death or accidental cell death or necrosis is the sort of “intuitive” death that scientists assumed centuries ago. That is, one that passively follows the end of life, without regulation. It is important to define necrosis carefully as in much of the literature, necrosis is loosely used to refer to non-apoptotic forms of cell death (Fink & Cookson, 2005). Indeed, necrosis is a type of death that is typically encountered under conditions of extreme catastrophic stress

(boiling temperatures, irreparable physical damage, exposure to supernatural concentrations of chemicals etc.) and characteristically involves the rupture of the cellular membrane; irreversibly and instantaneously equilibrating the internal and external milieus. Clinically, this poses problems for multicellular organisms as the leakage of intracellular contents into the extracellular environment can cause unwanted inflammation (Fink & Cookson, 2005). Morphologically, necrosis can be identified by a few key features. Cells that have died via necrosis are not packaged into smaller apoptotic bodies but appear "exploded". This appearance is in large due to the rupture of the plasma membrane and non-ordered breakdown of intracellular structures and organelles and leakage of intracellular content into the extracellular space. Using scanning electron microscopy, necrotic cells have also been seen to involve the formation of large cell surface invaginations or "bubbles" that precede the eventual rupture of the cell (Rello et al., 2005). On a molecular level, accidental death is also fundamentally different than regulated cell death. Accidental cell death occurs independently of the cell's response to stress. In other words, ACD or necrosis does not depend on any mechanism or process to happen; rather it is the direct result of extreme stress.

Experimentally, this distinction is often challenging as previously mentioned, many reports have defined any death that is caspase independent or void of apoptotic markers necrotic cell death (Fink & Cookson, 2005). This approach risks classifying non-apoptotic but regulated forms of cell death as necrosis. Traditionally, the diagnosis of necrosis is based on the loss of plasma membrane integrity and the absence of phosphatidylserine externalization which is normally detectable using fluorescent proves such as Annexin V (Clapp, Portt, Khoury, Sheibani, Eid, et al., 2012; Krysko et al., 2008). Additionally, necrosis is accompanied by the non-ordered breakdown of intracellular structures including the nucleus. Indeed, new techniques have aimed to capitalize on this using fluorescent markers for high mobility group 1 (HMGB1) which normally resides in the nuclear

envelope but reportedly translocates to the cytosol during necrosis (Paudel et al., 2018). Another aspect worthy of consideration is the timeline of necrosis. The death of a cell under extreme stress is often rapid if not instantaneous such as in extreme temperature stress. Such considerations are often missing from discussions on necrosis. The mechanisms that drive regulated cell death operate on a time scale of minutes to hours while necrotic deaths can occur within seconds (Green, 2005). Thus, in some instances, the observation and measurement of necrosis necessitates a degree of urgency to accurately capture the event.

1.3.4. Regulated Cell Survival (RCS)

In the same way that cells can deliberately choose to die via RCD via genetically encoded mechanisms, they are also capable of promoting their own survival through regulated cell survival (RCS). Indeed, relatively less is known about RCS than RCD. Much of what we understand about RCS is in the way of antagonizing pro-RCD pathways. In fact, the first and best understood example of regulated cell survival is the BAX inhibitor Bcl-2 protein (Bakhshi et al., 1985; Tsujimoto, 1998; Tsujimoto et al., 1984). Named for their discovery in B-cell lymphomas, Bcl-2 is overexpressed in several cancer types and a prototypical example of a tumorigenic factor. Bcl-2's activity during stress serves to inhibit BAX by directly binding it, preventing the onset of MOMP (Kale et al., 2018). Indeed, multiple mammalian cell studies have demonstrated that the expression of pro-survival Bcl-2 family members and/or the loss of pro-death members leads to stress resistance (R. Ardehali et al., 2011; Lucantoni et al., 2021). In the same vein, as seen in many cancer types, overexpression of Bcl-2 plays a tumorigenic role in maintaining cancer cell

populations while also increasing their resistance to chemotherapeutic stress (Lucantoni et al., 2021; Sochalska et al., 2015).

Interestingly, the overexpression of mammalian Bcl-2 in the budding yeast *Saccharomyces cerevisiae* also protects against stress-mediated cell death (Ligr et al., 1998; Polčic et al., 2015; Tao et al., 1997). Although eukaryotic, the yeast genome does not encode for a BAX homolog suggesting that Bcl-2 may play a more general protective role or in addition to its role in inhibiting BAX. Nonetheless, the antagonization of BAX remains an attractive target for RCS research as screens and experiments in yeast as well as mammalian cells continue to be performed to identify novel BAX suppressors (Clapp, Portt, Khoury, Sheibani, Eid, et al., 2012; Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012; Eid et al., 2016; Zhou et al., 2020). To this end, several novel BAX suppressors have been identified and characterized (Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012; Eid et al., 2016; Zhou et al., 2020). In many cases, both the yeast and mammalian orthologues have been shown to protect against BAX expression in both cell types (Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012; Eid et al., 2016). Surprisingly, many of the identified pro-survival sequences have also demonstrated protection against other stress like excess copper (Eid et al., 2016; Eid et al., 2014; Horowitz et al., 2013; Jones et al., 2015; Sheibani et al., 2015; Zhou et al., 2020). These findings may suggest that the molecular machinery driving stress-mediated cell death is more complex than just the activation of BAX.

In addition to inhibiting BAX, the inhibition of stress activated caspases also represents another critical point in the promotion of cell survival. In yeast studies, the inhibition or loss of the metacaspase YCA1 has been shown to increase cell tolerance to various stress including those that increase ROS (Bettiga et al., 2004; Guaragnella et al., 2006). Interestingly, the use of caspase inhibitors in mammalian studies has also been reported to promote caspase-independent modes of

cell death (Vandenabeele et al., 2006). Interestingly, while there exist many pharmacological caspase inhibitors, few are in clinical trials (Dhani et al., 2021). This may again highlight the pleiotropic nature of RCD machinery and the need to expand our models of stress beyond the accumulation of ROS.

Nonetheless, the accumulation of intracellular ROS is another routinely examined marker for both the induction of stress as well as apoptosis due to their role in oxidative stress. Much effort has gone into understanding how the levels of intracellular ROS relate to cell viability. Exogenously added hydrogen peroxide is a common agent used to induce oxidative stress and cell death (Chen et al., 2009; Wei et al., 2000). Cells treated with increasing doses of hydrogen peroxide have been shown to have significantly increased levels of intracellular ROS as well as decreased viability (Chen et al., 2009; Wei et al., 2000). In experiments in yeast with excess metal stress such as copper, cells also show elevated intracellular ROS in addition to significantly decreased viability (Eid et al., 2016; Zhou et al., 2020). In the same vein, the ectopic expression of pro-survival sequences in these yeast have been shown to rescue viability alongside a significant reduction in intracellular ROS (Eid et al., 2016; Zhou et al., 2020). These findings are consistent with the oxidative stress model that suggests that ROS plays a driving role in the promotion of cell death. Thus, anti-oxidants have received a significant amount of attention as a strategy to promote both cellular and physiological health. Together, such studies have ultimately driven the formation of the multi-billion-dollar anti-oxidant industry; primarily focused on the this correlation. Interestingly, despite in vitro successes with anti-oxidant strategies in reducing stress and increasing viability, there is a surprising lack of anti-oxidant therapies available as most clinical trials have been negative in the context of chronic therapy (A & A, 2018; Steinhubl, 2008). Such irony likely suggests that while important, ROS is necessary but insufficient for RCD processes

and is more likely a single component of a much more complex stress signalling network (Zhou, Eid, Boucher, et al., 2019).

1.3.4.1. Autophagy

A unique survival strategy available to cells is autophagy. Autophagy is a conserved process that serves to degrade unnecessary or defective components via a lysosome-dependent mechanism (Ichimiya et al., 2020; Khandia et al., 2019). Autophagy is typically triggered in response to nutrient deprivation or starvation in order to breakdown cellular components and recycle important macromolecules (Ichimiya et al., 2020; Khandia et al., 2019). In this way, autophagy can serve to prevent starvation and promote the survival of a cell under stress. Autophagy is regulated by the autophagy (ATG) gene family; originally discovered in yeast (Takeshige et al., 1992; Tsukada & Ohsumi, 1993). These ATG genes and their orthologues serve to regulate the formation and activity of specialized lysosomes called autophagosomes that capture and catabolize non-essential cellular material into useable macromolecules. In yeast knockout experiments, cells lacking ATG1 and ATG7 are unable to mount autophagic responses to stress (Cebollero & Reggiori, 2009). Specifically, these cells are unable to form autophagosomes necessary for autophagy to occur and are subsequently more sensitive to starvation stress (Cebollero & Reggiori, 2009). Interestingly, in studies with *Drosophila*, prolonged activation of autophagy genes robustly activates autophagy but also increases cell death (Scott et al., 2007). Such evidence suggests that autophagy is a protective process that in large maintains the survival of cells under stress in a way that does not necessarily antagonize a specific RCD process itself

but rather attempts to alleviate stress by promoting homeostasis but nonetheless has its limits and may eventually promote cell death.

1.3.4.2. Heterologous Expression of Pro-Survival Genes

Experimentally, stress-mediated RCD responses in yeast can also be prevented by the overexpression of pro-survival or anti-RCD genes (Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012; Eid et al., 2014; Horowitz et al., 2013; Jones et al., 2015; Sheibani et al., 2015). In mammalian cell models, the prevailing explanation for the anti-RCD or pro-survival property of Bcl-2 is through the direct binding and inhibition of stress-activated BAX protein. However, the heterologous expression of Bcl-2 in transgenic animals such as yeast also confers pro-survival advantages despite cells lacking direct Bax orthologues (Kane et al., 1993; Trancíková et al., 2004). Additionally, the ectopic expression of Bax in yeast also leads to RCD that can be protected against by the heterologous expression of Bcl2 as well as pro-survival sequences. Thus, it is likely that this points to a more ancient and conserved RCD network that is shared between species.

Owing to the facile experience of working with yeast, cell death screens of cDNA expression libraries in yeast expressing mammalian BAX have been used to identify novel suppressors (Clapp, Portt, Khoury, Sheibani, Eid, et al., 2012). Through this, several human genes as well as their yeast orthologues have been shown to protect against several stresses including exogenous copper, BAX expression, hydroxyurea, iron and rapamycin (Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012). The role of these genes are highly varied and include lactate dehydrogenase b, 14-3-3β/α, ferritin, and ribosomal protein L9 to name a few. Although the exact pro-survival mechanism of action is unknown for many of these sequences, significant reductions

in intracellular ROS under stress and increases in relative viability are associated with virtually all of them.

1.4. Humanized Yeast Models of Cell Death

The eukaryotic yeast *Saccharomyces cerevisiae* offers unique advantages to the study of many different aspects of cell biology including cell death processes. Indeed, many regulated cell death processes are conserved in yeast but interestingly, yeast lack apparent Bcl-2 family member orthologues (Polčic et al., 2015). Nonetheless, yeast are excellent models for the study of Bcl-2 family proteins (Polčic et al., 2015). The ability of mammalian BAX expression to cause cell death in yeast was initially observed in yeast two-hybrid studies that tried to examine the interactions between BAX and Bcl2 (Sato et al., 1994). Shortly after, studies emerged that clarified the lethal phenotype of BAX expression in yeast and linked it to the same phenotypical changes observed during mammalian apoptosis (Ligr et al., 1998). This included plasma membrane blebbing, chromatin condensation and DNA fragmentation. These data suggested that the heterologous expression of BAX relied on a conserved framework even in lower eukaryotes to carry out its pro-death functions. In fact, as in mammalian cells, BAX-mediated cell death in yeast also involves regulated disruption of the mitochondrial membrane via the insertion of BAX and the release of cytochrome-c. Furthermore, in each of these studies, the co-expression of the BAX inhibitor Bcl-2 prevented these effects along with other pro-survival Bcl2 family members including Bcl-xL and Mcl-1 (Ligr et al., 1998). Taken together, these data suggest that despite not having direct orthologues, it is possible that Bcl-2 proteins operate on a conserved framework that is retained in yeast (Carmona-Gutierrez et al., 2010; Galluzzi et al., 2018).

1.5. Rational for Thesis & Objectives

The relationship between cellular stress and cellular response is fundamentally important to understanding how cells live in their environments. Virtually all diseases are related to the interaction between cells and stress. In fact, the death or survival of cells under stress often have serious implications for the survival and health of multicellular organisms including humans. Despite decades of research, we are far from an exhaustive understanding of the mechanisms that drive cells towards both their survival and death. Even less is known about the ways in which cells can regulate their own survival in response to stress. Till now, our understanding of regulated cell death and cell survival have largely been built upon our models and understanding of apoptosis; a type of RCD. Oxidative stress and caspase activation are both commonly discussed in these models of RCD and RCS. However, while these models are successful at describing many cell stress scenarios, they fail to ubiquitously capture all stress contexts and translate into effective targeted therapies for pathological cell death/survival. This is evident in studies that have aimed to inhibit BAX, ROS, and caspases, suggesting that a more general re-examination of the relationship between cellular stress and cell death and/or survival is necessary.

The goal of this thesis is to firstly contribute further to the understanding of stress and stress responses relationships by investigating the balance between the regulatory mechanisms that promote the death or survival of a cell using yeast as a model. The use identification of and characterization of novel pro-survival sequences serves to expand our understanding of the general pro-survival network in cells as well as serving as tools to study survival response in yeast under stress. This thesis also aims to challenge and re-explore fundamental tenets regarding the relationship between stress and stress responses in yeast.

The specific objectives of this thesis are:

1 To investigate typical stress responses in yeast and how they can be modulated using pro-survival sequences.

2 Characterize the pro-survival potential of a novel Bax suppressor human CSRP3.

3 Utilize pro-survival sequences as tools to investigate novel RCD/RCS pathways.

4 Critically re-evaluate the relationship between the dose or intensity of cellular stress and matching cellular responses in different stress contexts.

Chapter 2.

Materials and Methods

2.1 Yeast strains and Plasmids

The *Saccharomyces cerevisiae* yeast strain BY4742 (MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was used as the wild-type strain in all experiments and all mutants used were isogenic to BY4742. Plasmids containing the cDNAs for BAX, 14-3-3 and CSRP3 under the control of the galactose inducible *GAL1* promoter were as described previously (Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012; Eid, Zhou, et al., 2017; Yang et al., 2006). Plasmids expressing the yeast ORFs for *RGA1*, *RGA2*, *LRG1* and *PXL1* were also expressed under the control of the *GAL1* promoter using a *URA3* selectable marker (Jones et al., 2015).

2.2. Yeast growth and maintenance

Yeast cells were routinely grown and maintained in synthetic minimal media consisting of yeast nitrogen base (YNB), 2% glucose and the required amino acids or bases. To induce gene expression of sequences contained within plasmids, glucose was replaced with galactose in media consisting of 2% galactose and 1% raffinose (Eid et al., 2016; Eid, Zhou, et al., 2017). Yeast cells harbouring the aforementioned plasmids were transformed using lithium acetate and routinely selected for and maintained in YNB glucose media excluding uracil.

2.3. Growth Assays

To assess the growth of each strain in the presence and absence of stress, spot assays were used. Cells were grown overnight in YNB glucose media to saturation and 100uL of freshly

saturated cultures of each strain were then diluted in fresh YNB galactose media and further incubated for 4h at 30 °C with shaking to induce GAL1 gene expression. Relative to yeast grown in fresh YNB glucose media, there is a noticeable lag in the re-growth of cells placed in YNB galactose media. Nevertheless, there are no discernable differences in growth between yeast transformed with empty vector or that with CSRP3 or 14-3-3. Cultures were then serially diluted five-fold with sterile water and aliquots (5uL) were spotted onto nutrient agar media prepared with and without exogenously added stress. These nutrient agar plates were then incubated for 72 h at 30 °C and subsequently photographed. The different concentrations of all stressors were added directly into the nutrient agar media before pouring the plates. All spot assays were performed a minimum of three times with similar results.

2.4. Viabiltiy Assays

To assess viability, freshly saturated cultures of each yeast strain were grown in YNB glucose, diluted in YNB galactose media and further incubated for 4h at 30 °C with shaking to induce gene expression. Cells were then incubated for an additional 18–20 h at 30 °C with shaking in YNB galactose with or without indicated concentrations of stress. Cells were stained with the vital dye trypan blue and then microscopically examined. A minimum of 300 cells were scored for each culture. Data obtained from viability experiments are shown as the mean ± standard deviation of triplicate experiments repeated a minimum of three independent times. Statistical significance of the data was determined using a Student *t*-test.

2.5. ROS Detection Assays

To detect changes in the levels of intracellular ROS, freshly saturated glucose-grown cultures were diluted in YNB galactose media and further incubated for 4h at 30 °C with shaking to induce GAL1 gene expression. Cells were then incubated for another 4 h in the presence or absence of stress. Subsequently, dichloro-dihydro-fluorescein diacetate (DCFH-DA) was added to each culture to a final concentration of 20 μg/mL. The cultures were then incubated for an additional 2h, washed twice with sterile water and then visualized using fluorescent microscopy through a FITC optical filter. Photographs of the resulting images were taken.

2.6 Site Directed Mutagenesis

To generate mutations in the LIM domains of CSRP3,

Primers were designed to create two mutants using site-specific mutagenesis that was used in a rolling circle amplification polymerase chain reaction (RCA-PCR). Two primer sets were designed using the Kit QuickChange II from Agilent Technologies (cat#2005230) to make the necessary point-mutations to the original nucleotide sequence of CSRP3 (NM_003476.5). The first mutation targets C58 and replaces it with a glycine residue. C58G is a clinically relevant and published mutation that disrupts the zinc-coordination of LIM1 domain. The second mutation, E118STOP introduces a premature stop codon before LIM2, resulting in a truncated protein containing a single LIM domain. To create the C58G mutant variant of CSRP3, the following primers were used: 5'-gagtcggagatctacGgcaaggtgtgctatg – 3' and 3' – cataggcacaccttgCcgtagatctccgatc – 5'. To create the E118STOP mutant, the following primers

were used: 5’ – aagtttggagagtccTagaagtgccctcgat – 3’ and 3’ – atcgagggcacttctAggactctccaaactt – 5’.

The mutagenesis protocol was carried out using the QuikChange II-E site-directed mutagenesis kit from Agilent Technologies. Strand synthesis was carried out for the control (pWhitescript 4.5-kb control plasmid), CSRP3^{C58G} (10ng and 25ng), and CSRP3^{E118X} (10ng and 25ng). The pWhitescript control plasmid was used to test the efficiency of the mutant plasmid generation (mutagenesis control). To complete the reaction, 1ul of PfuUltra HF DNA polymerase was added into each tube. Then, 1ul of the Dpn1 restriction enzyme was added to the control and four experimental reactions and were immediately incubated at 37˚C for 1 hour to complete the digestion of the parental strand. Before the 1-hour incubation was complete, 50ul of DNA competent XL1-Blue E. coli cells were incubated on ice with the Dpn1-treated DNA and a standard heat-shock method was used to facilitate the transformation according to the manufacturer (Agilent Technologies). These cells were then incubated at 37˚C with media for 1 hour with shaking at 250 rpm. After the incubation, 100ul and 250ul of each reaction were spread onto to two different LB-ampicillin agar plates and incubated at 37˚C for 16 hours.

Chapter 3.

Results

3.1. Modulating Stress Responses in Yeast with Novel Pro-Survival Sequences

Previous studies in our lab have used yeast cells undergoing RCD due to the heterologous expression of mammalian BAX to directly screen for and identify pro-survival sequences in a human heart cDNA expression library (Clapp, Portt, Khoury, Sheibani, Eid, et al., 2012). Here, we report on the identification and characterization of a novel BAX-supressing human 850 bp cDNA sequence isolated from this screen. The examination of the amino acid sequence deduced from the nucleotide sequence of the cDNA is an exact match to the sequence encoding human cysteine and glycine rich protein 3 (CSRP3; GenBank accession no. NP_003467.1).

3.1.1. Human CSRP3 is a LIM-Containing Sequence that can Modulate Yeast Stress Responses

To confirm the BAX-suppressing ability of the CSRP3 cDNA, wild-type yeast were doubly transformed with mammalian BAX in combination with empty vector, a previously characterized BAX suppressor human 14-3-3β/α, or CSRP3 under the control of the yeast inducible GAL1 promoter. These transformants were grown in liquid media, serially diluted and aliquoted onto nutrient agar media in spot assays (Fig. 2A). All three strains grew normally and showed no discernible differences when grown on glucose. On galactose inducible media, growth was not observed for the double transformants harbouring both empty vector and the BAX-expressing vector. In contrast, cells expressing BAX and co-expressing 14-3-3β/α or CSRP3 grew significantly more than control (Fig. 2A). This confirmed that the expression of CSRP3 was

sufficient to suppress the deleterious effects of BAX in yeast. Previously characterized suppressors of BAX from this screen including 14-3-33β/α were found to protect against other stresses unrelated to BAX (Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012). To determine if CSRP3's protective characteristics when expressed were specific to BAX, we examined the effects of copper stress on CSRP3-expressing cells. Copper is a commonly used extracellular stress capable of inducing RCD in both mammalian and yeast cells and can be protected against by expressing pro-survival sequences including 14-3-3β/α (Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012). Spot assays were performed with wild type yeast singly transformed with empty vector, 14-3-3β/α or CSRP3 on nutrient agar media containing RCD inducing levels of copper (Fig. 2B). Yeast cells expressing CSRP3 or 14-3-3β/α were more resistant to the deleterious effects of copper stress compared to empty vector (Fig. 2B). To determine if the protection conferred by the expression of CSRP3 was related to cell death and not just growth inhibition, it is recommended that cell death be measured directly (Galluzzi et al., 2018). To this end, we performed viability assays by microscopic examination of cells stained with the vital dye trypan blue to monitor the lethality in response to copper stress of each strain. Yeast transformants harbouring empty vector, 14-3-3 β/α or CSRP3-expressing vectors showed close to 100% viability when grown on galactose inducible media (Fig. 2C). In contrast, yeast transformants expressing CSRP3 or 14-3-3β/α had a significantly higher proportion of live cells (54.3% ± 4.9 and 82.4% ± 2.7 respectively) compared to empty vector (31.2% ± 7.6) after 18 h of growth in the presence of copper. These results indicate that the expression of CSRP3 protects against copper-induced RCD.

The cellular response to BAX and many other stresses in yeast is pleiotropic and often includes not only the induction of RCD but also the inhibition of cell growth as well as the activation of autophagy (Portt et al., 2011). In myotubes, the protective effect of CSRP3 has been

associated with its ability to regulate autophagy (Rashid, Runci, Polletta, et al., 2015). To examine this possibility in yeast, we expressed CSRP3 in an ATG1Δ mutant that is unable to carry out macro-autophagy. As shown using spot assays, mutant and wild type cells expressing CSRP3 are similarly protected from the effects of copper (Fig. 2D). This indicates that autophagy activation may not play a major role for CSRP3 protection in yeast.

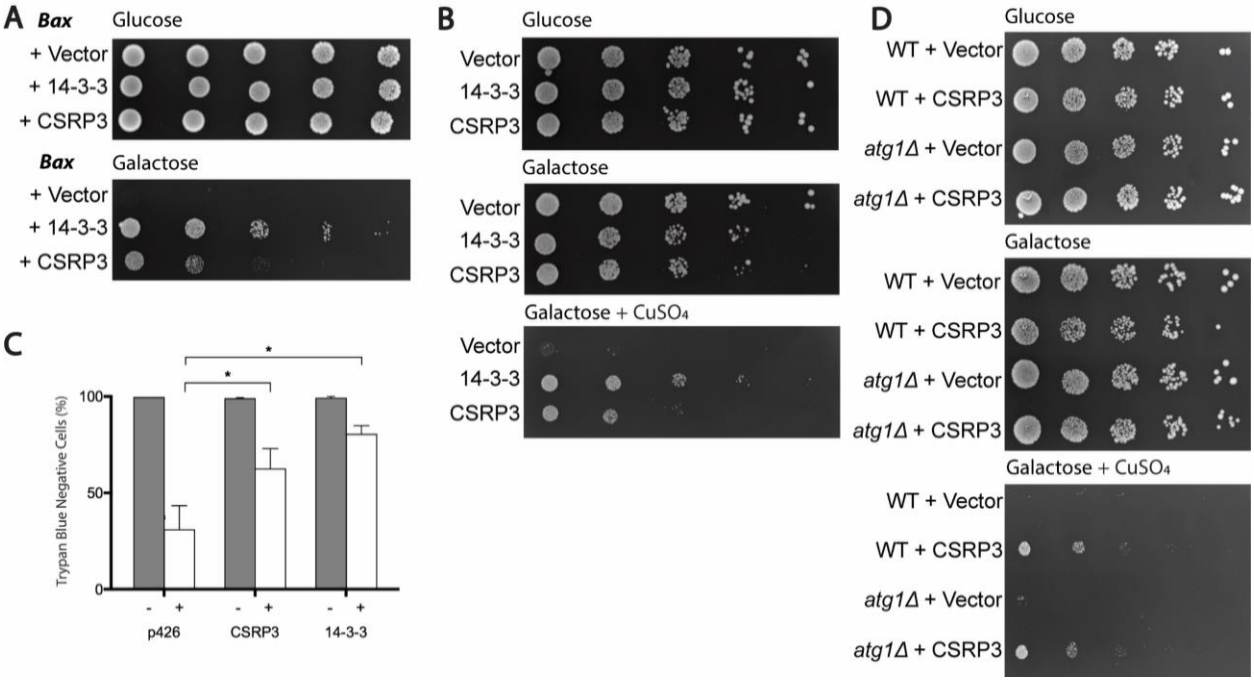

Figure 2. ***Human CSRP3 is a BAX suppressor and pro-survival sequence that prevents copper mediated Regulated Cell Death. A)*** *Wild type BY4741 cells were doubly transformed with a murine BAX plasmid expressing plasmid in combination with an empty vector or with a vector expressing a 14-3-3 encoding cDNA or with a vector expressing a CSRP3 encoding cDNA under the control of theGAL1promoter. The double transformants were selected and maintained by the omission of uracil and histidine from the growth media. Freshly saturated YNB glucose grown cultures of the transformants grown in selectable were subcultured into fresh YNB galactose media*

and incubated with shaking at 30 °C for 4 h. The cultures were then serially and sequentially diluted (1:5) using sterile water and 5 μL aliquots of each dilution were spotted into YNB media agar plates with glucose or with galactose. The plates were incubated at 30 °C for 3 days and photographs of the results are shown. ***B)*** *C Wild type BY4741 cells were transformed with a single plasmid consisting of either the empty vector or a vector expressing human 14-3-3 or CSRP3. B, Freshly saturated cultures of the transformants were subcultured into YNB galactose media (1:5) and allowed to grow for 4 h at 30 °C with shaking. The cultured were then serially diluted and aliquots were spotted onto YNB nutrient agar plates containing glucose, galactose alone or galactose and 2 mM copper sulphate (CuSO4). The plates were incubated at 30 °C for 3 days and photographed.* ***C)*** *Freshly saturated cultures of the three different transformants (empty vector, 14-3-3 and CSRP3) were inoculated into fresh YNB galactose media (1:100) and incubated at 30 °C for 4 h. Each of the cultures were then separated into two, with one getting 1.2 mM copper sulphate (+CuSO4) while the other received no addition (−CuSO4). The cultures were further incubated for 18 h and examined microscopically after staining with the vital dye trypan blue. Cells that stained blue were scored as dead while clear cells were scored as viable. We examined a minimum of 300 cells per culture and the experiments were repeated at least three times. The data is shown as the mean ± standard deviation of triplicate experiments repeated a minimum of three independent times. *, indicates significant differences, using a Student t-test, between control cells (Vector) cells expressing 14-3-3 or CSRP3 that were treated with copper ($p < 0.001$). D, Spot assay using wild type (WT) as well as atg1Δ mutant cells transformed with a single plasmid consisting of either the empty vector or a vector expressing human CSRP3 essentially as shown in B (see above).*

Cellular response to stress is commonly marked by an increase in reactive oxygen species (ROS) (Zhou, Eid, Miller, et al., 2019). We used the cell permeable agent DCFH-DA that is oxidized to a fluorescent chromophore in the presence of ROS-generating molecules to directly detect increases in ROS in stressed cells. Cultures harbouring empty vector were observed to contain a small level of ROS positive cells which were observed microscopically as fluorescent cells. The number of fluorescent cells that could be visualized increased sharply after 4 h copper challenge (Fig. 3). In contrast, yeast expressing CSRP3 or 14-3-3β/α had significantly less visibly detectable ROS positive cells in both the presence and absence of stress. These findings suggest that CSRP3 protects against copper-induced stress in part by mitigating the accumulation of ROS. Taken together, these results indicate that CSRP3 is a pro-survival sequence capable of promoting the survival of yeast challenged with RCD-inducing stresses. These findings align with previous reports on the protective role of CSRP3 in regulating RCD as well as autophagic responses in skeletal muscle cells. Our results suggest that CSRP3 may participate in RCD processes that are conserved in both mammalian and yeast cells to regulate survival.

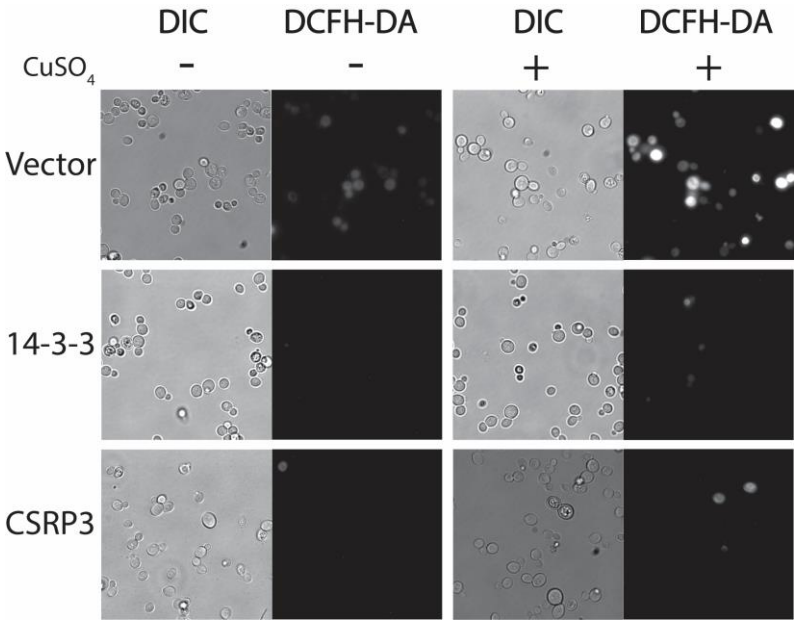

Figure 3. ***CSRP3 decreases copper mediated increases in ROS.*** *Wild type cells transformed with a single plasmid consisting of either the empty vector or a vector expressing human 14-3-3 or CSRP3 were used to monitor ROS levels produced by copper stress. The cells were treated without (−) or with copper (+CuSO4) for 2 h. The cultures were then incubated for an additional 2 h with DCFH-DA and the cells were examined by light (DIC) and fluorescent light (DCFH-DA) microscopy. Representative photographs are shown.*

3.1.2. LIM domains are present in CSRP3 as well as four yeast proteins

We next asked how CSRP3 might mediate its pro-survival effects in yeast challenged with RCD-inducing stresses. Since structure is related to function, we first examined the amino acid sequence of CSRP3. In silico analysis shows that CSRP3 is a "LIM-only" sequence composed of two LIM domains, glycine-rich regions and a nuclear localisation sequence (NLS) (Fig. 4A). LIMs are protein structural/functional domains consisting of two tandemly repeated zinc fingers (Kadrmas & Beckerle, 2004). Unlike GATA-binding zinc fingers, these domains are reported to mediate protein-protein interactions including but not limited to dimerization with other LIM domains. The presence of the LIM domain in the human genome is widespread and found in over 50 genes (Kadrmas & Beckerle, 2004; Koch et al., 2012). Despite its prevalence, the lack of proper functional assays has left our understanding of LIM function including specific binding partners obscure. LIM containing proteins often contain other functional domains including a homeobox or kinase domain and these confer specific functions on the protein. In this instance, CSRP3 is a LIM-only domain member of the LIM containing superfamily of proteins (Kadrmas & Beckerle, 2004). This led us to postulate that the LIM domain is functionally responsible for the pro-survival

properties of the heterologously expressed CSRP3 in yeast. To investigate this further, we first looked for other LIM-containing sequences in the yeast genome using standard BLAST algorithms. The yeast genome encodes four LIM domain-containing sequences, namely RGA1, RGA2, LRG1 and PXL1 (Fig. 4B). All four yeast LIM proteins are involved in the regulation of stress responses in yeast including osmotic shock, pheromone, starvation, and cell wall integrity responses (Jolly & Morimoto, 2000; Nabiilah, 2021; Russell, 2003). Since these endogenous yeast LIM genes encode for regulators of stress response pathways, we hypothesized that CSRP3, through its LIM domains specifically enhances or interferes with endogenous yeast LIM proteins to generate a pro-survival response (Fig 5). Alternatively, the LIM domains may serve to promote pro-survival responses by directly interacting with and modulating the function of pro- and/or anti-RCD sequences.

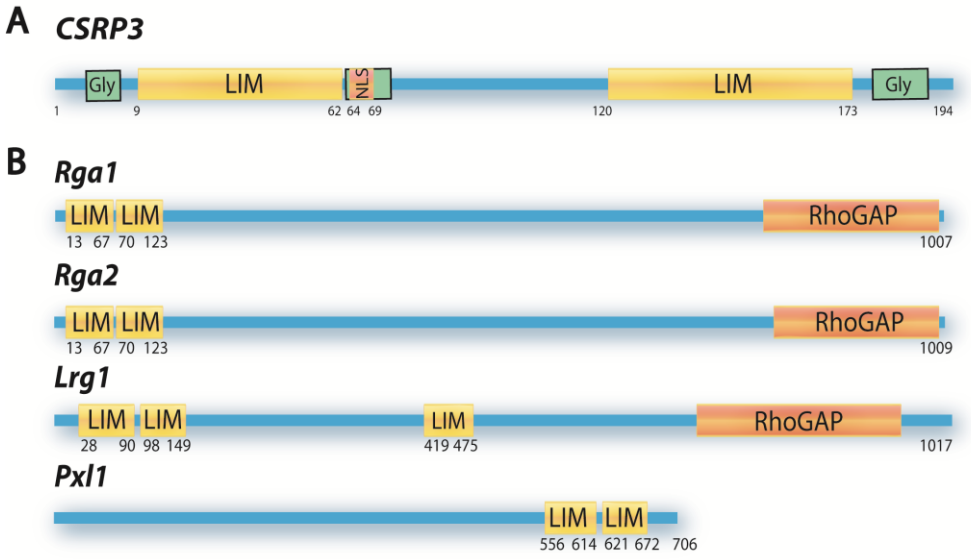

Figure 4. ***Schematic representation of the structure of human CSRP3 and of the LIM domain containing yeast proteins as well as a model depicting their possible functional interactions. A)***

A linear depiction of the predicted CSRP3 protein structure showing the location of its structural domains including the glycine rich regions (Gly), a putative Nuclear Localization Sequence (NLS) as well as the LIM domains. The locations of the different domains are numbered within the 194 residue CSRP3protein. ***B)*** *Schematic representation of the four yeast LIM containing proteinsRga1, Rga2, Lrg1and Pxl1. The locations of the recognized functional LIM RhoGAP domains are shown.*

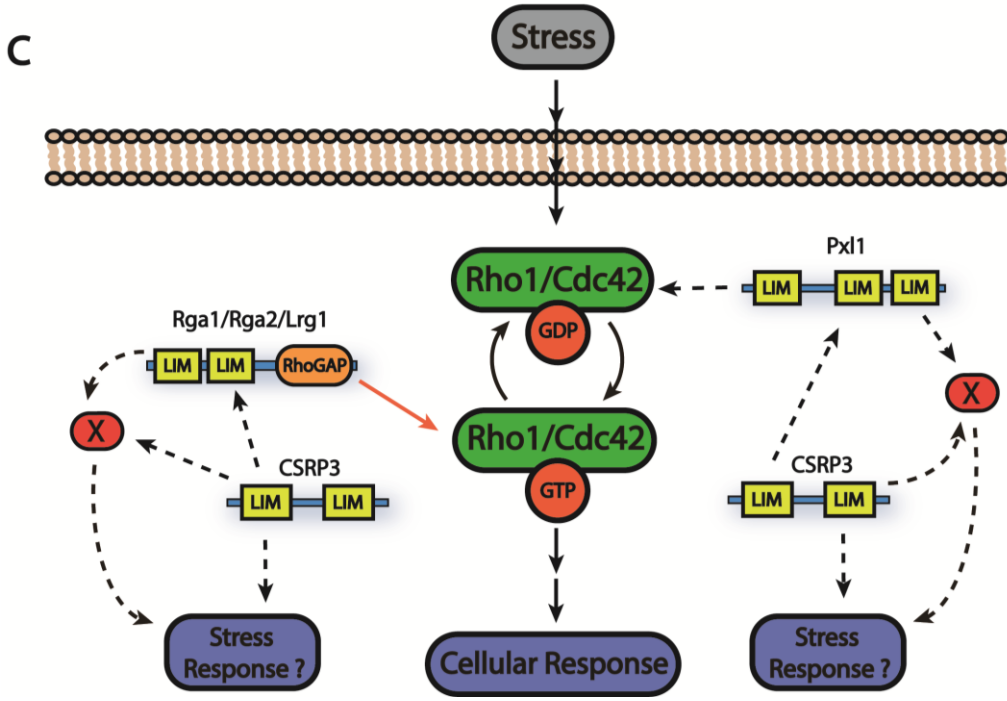

Figure 5. ***Testable model showing the functions of the yeast LIM proteins possible functions by which human CSRP3 may prevent stress mediated RCD.*** *Yeast LIM proteins are reported to functionally interact with the GTPase Rho or Cdc42. The small G proteins become GTP-bound when activated by stress (or stimuli such as pheromone). The GTP-bound G-proteins then serve to activate specific cellular responses by initiating MAP kinase signalling cascades (not shown).*

The LIM containing proteins Rga1p, Rga2p and Lrg1p contain a RhoGAP (GTPase Activating or Accelerating) domain that serves to stimulate the GTPase activity of the small G proteins. This serves to accelerate the hydrolysis of the bound GTP to GDP and thus inactivate the G-protein. Pxl1p on the other hand is reported to act as a GDI (Guanine Dissociation Inhibitor) and thus serve to prevent the activation of small G proteins by reducing its ability to replace GDP with GTP. CSRP3 through its LIM domains may serve as a dominant form of the yeast LIM proteins by interacting with the putative target of the yeast LIM protein (shown as "X") or with the yeast LIM proteins themselves. Alternatively, CSRP3 may directly interact with other cellular constituents to promote cell survival in a process that in independent of the yeast LIM proteins.

3.1.3. Stress responses in cells overexpressing yeast LIM proteins

Although the yeast LIM domain containing proteins are involved in regulating stress responsive signalling pathways, their potential role as anti-RCD sequences have not been investigated. Furthermore, the specific role of the different LIM domains in any of these yeast proteins has also not been investigated. As a first step in analyzing LIMs in RCD responses, we generated yeast transformants harbouring the plasmids expressing the four different yeast LIM sequences (*RGA1*, *RGA2*, *LRG1* and *PXL1*) under the control of the *GAL1* inducible promoter (Fig 6). On glucose containing media, all transformants including the controls harbouring empty vector as well as the 14-3-3 β/α and CSRP3 expressing vectors showed similar growth (Fig 6A). In contrast, on *GAL1* inducible media alone in the absence of stress, there was an almost complete inhibition of growth in cells harbouring the plasmid expressing *RGA1* while cells with the *PXL1* expressing vector showed a moderate level of growth inhibition (Fig 6B). Global

analysis of the growth phenotypes of yeast cells over-expressing all the yeast ORFs had previously detected the effects of over-expressing *RGA1* but not the milder defect in cells with *PXL1* overexpression (Yoshikawa et al., 2011). The lack of growth associated with overexpression is a commonly observed phenotype that is largely thought to be due to non-specific stress. It is commonly observed with many other yeast sequences (Eid et al., 2014). The growth of cells harbouring the expression vectors for *RGA2* and *LRG1* were similar to the growth of control (Fig 6B). Spot assays on plates containing copper showed that the cells harbouring either the 14-3-3 β/α or the CSRP3 expressing vectors grew better than the empty vector control cells. In contrast, cells harbouring yeast LIM expressing constructs did not grow better on copper containing media. These data indicate that LIM containing proteins are not likely to be directly involved in attenuating stress mediated responses.

Due to their involvement in modulating Cdc42p and Rho1p responses, the yeast LIM containing proteins and their associated signalling pathways have been previously analyzed for their responses to several stresses not commonly associated with the induction of RCD in yeast. One commonly used stress is the non-ionic detergent SDS that is reported to activate the cell wall integrity pathway (Popolo et al., 2001). The addition of SDS at a concentration of 0.0075% leads to a noticeable effect on the growth of control cells (Fig 6B). Cells harbouring the pro-survival 14-3-3 β/α or CSRP3 showed similar growth inhibition in response to the SDS. Thus, SDS appears to induce a form of cell death that differs from the preventable RCD induced by copper and BAX expression. Similarly, there was no protective effect due to the overexpression of any of the yeast sequences (Fig 6B). As observed above with copper stress (Fig 6A) only RGA2 cells showed significant growth with added of SDS. The inability to grow with copper and SDS is especially

noticeable with the cells harbouring the LRG1 expressing plasmid since this transformant grows normally on galactose inducible media alone.

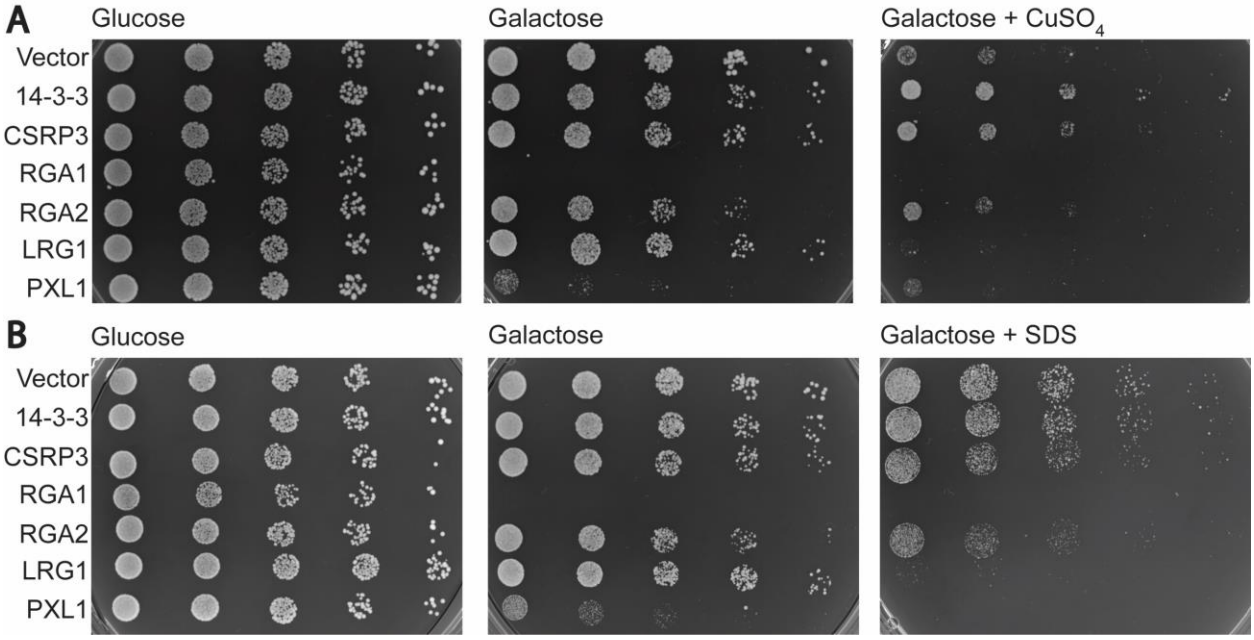

Figure 6. ***Yeast LIM proteins do not protect against copper or SDS mediated cell death.*** *Wild type yeast transformants harbouring plasmids expressing, under the control of the* GAL1 *promoter, the yeast LIM containing sequences (*Rga1, Rga2, Lrg1 *and* Pxl1*) as well as cDNAs for human 14-3-3 were analyzed using the spot assay. Aliquots of serial dilutions of cultures of the different transformants were spotted onto YNB nutrient agar plates containing Glucose, Galactose or A, Galactose and copper sulphate ($CuSO_4$) and B, Galactose and SDS. The plates were incubated for 3 days and then photographed.*

3.1.4. Analysis of stress responses in yeast LIM mutants

The overexpression of anti-RCD sequences does not always lead to stress resistant phenotypes (Portt et al., 2011). In these cases, the knockouts of these genes can be useful for uncovering potential pro-survival functions. Indeed, the reduction in the levels of a pro-survival protein has been reported to lead to an increased sensitivity to RCD inducing stress (Portt et al., 2011; Zhou, Eid, Boucher, et al., 2019). To further assess potential anti-RCD roles, we analyzed the effects of RCD inducing copper on yeast strains lacking the four different LIM encoding genes (Fig 7). The addition of 2 mM copper to the media of control (WT) cells leads to a predicted inhibition of growth (Fig 7A). With the exception of the *pxl1*Δ strain, there is no large difference in the growth between LIM gene knock-out strains (*rga1*Δ, *rga2*Δ and *lrg1*Δ) compared to the wild type strain with 2 mM copper (Fig 7A). This indicates that these three yeast LIM genes are not likely to have general pro-survival functions.

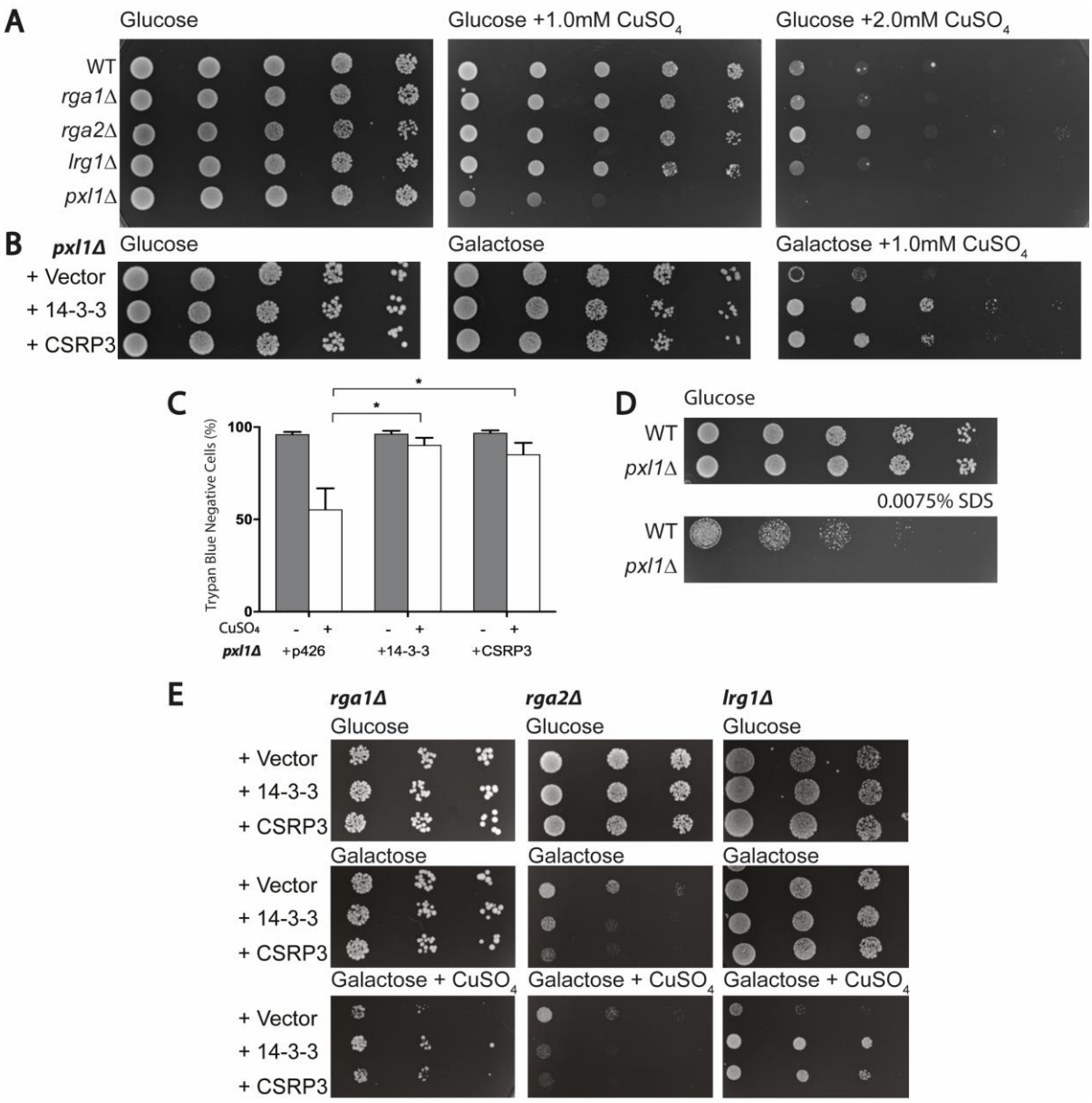

Figure 7. ***Analysis of copper sensitivity of yeast LIM gene knock outs reveals that*** **pxl1Δ** ***is synthetically lethal with sublethal copper***. ***A)*** *Wild type (WT) as well as the isogenic strains with gene knock-outs for the LIM encoding genes (*RGA1, RGA2, LRG1 *and* PXL1*) were analyzed by spot assay on YNB agar media plates containing Glucose, Galactose and 1.0 mM* $CuSO_4$ *and Galactose and 2.0 mM* $CuSO_4$*.* ***B)*** *Transformants of the strain lacking the LIM encoding* PXL1 *gene harbouring empty vector or the vectors expressing 14-3-3 or CSRP3 were*

analyzed by spot assay. Serially diluted aliquots of cultures of the different transformants were spotted onto YNB nutrient agar plates with Glucose, Galactose or Galactose and 1.0 mM $CuSO_4$. ***C)*** *The transformants of the strain lacking the LIM encoding* PXL1 *gene harbouring empty vector or the vectors expressing 14-3-3 or CSRP3 were analyzed for viability using microscopical examination of trypan stained cells. The transformants were treated with $CuSO_4$ and viability was examined after 20 h. As described in the legend to Fig. 1, cells that stained blue were scored as dead while clear cells were scored as viable. We examined a minimum of 300 cells per culture and the experiments were repeated at least three times. The data is shown as the mean ± standard deviation of triplicate experiments repeated a minimum of three independent times. *, indicates significant differences, using a Student* t*-test, between control cells (Vector) cells expressing 14-3-3 or CSRP3 that were treated with copper ($p < 0.001$).* ***D)*** *Wild type (WT) and the* PXL1 *gene knock-out strains were analyzed by spot assay on YNB media plates containing Glucose and Galactose with SDS.* ***E)*** *Transformants of the strains lacking the LIM encoding* RGA1, RGA2 *and* LRG1 *genes harbouring empty vector or the vectors expressing 14-3-3 or CSRP3 were analyzed by spot assay. Serially diluted aliquots of cultures of the transformants were spotted onto YNB nutrient agar plates with Glucose, Galactose or Galactose and $CuSO_4$ (2 mM). All the plates in figure were incubated for 3 days at 30 °C and photographed.*

3.1.5. CSRP3 does not require endogenous yeast LIM proteins to protect against copper stress

LIM domains are thought to be able to interact with the LIM domains of other proteins (Kluska et al., 2018; Xu et al., 2017). It thus remains that CSRP3 may interact with endogenous LIM

containing proteins in yeast to generate a pro-survival effect. This interaction may lead to the observed copper resistance possibly by activating or inhibiting the yeast LIM proteins (Fig 5). To examine this, we used spot assays to determine the effect of copper on the growth of yeast strains lacking LIM genes but transformed with empty vector or vectors containing human 14-3-3 or CSRP3 (Fig 7). As observed, both 14-3-3 and CSRP3 could prevent the effect of copper on strains lacking *RGA1* and *LRG1* (Fig 7E) and as shown above for *PXL1* (Fig 7B). The results obtained with *rga2*Δ differ since CSRP3 could not protect it from copper stress (Fig 7E). This does not appear to be specific since the general pro-survival sequence 14-3-3 itself was also not able to protect the strain (Fig 7E). We do not understand the significance of these results but they may indicate a true specific synthetic lethality between *rga2*Δ and copper.

3.1.6 *pxl1*Δ is synthetically lethal with sublethal copper

In contrast to the other three LIM gene knock out strains, the cells lacking *pxl1*Δ showed complete growth inhibition with 2 mM copper. To further examine the extent of this hypersensitivity, we carried out a spot assay using a sublethal concentration of copper sulphate (1 mM). As expected, there is no copper mediated growth inhibition with the WT or with the *rga1*Δ, *rga2*Δ and *lrg1*Δ deletion strains (Fig 7A) In contrast, although there is growth of the *pxl1*Δ strain, copper nevertheless still inhibits its growth. This indicates that cells lacking *pxl1*Δ are hypersensitive to copper.

3.1.7 The synthetic lethality of *pxl1*Δ-subletahl copper results in non-specific activation of RCD

Yeast cells lacking a functional *PXL1* gene have no overt growth defects (Fig 7A). Yet these cells are hypersensitive to normally sublethal levels of copper (Fig 7A). It is possible that the combination of sublethal copper and the loss of *Pxlp* (*pxl1*Δ) is a case of synthetic lethality in which two individually non-deleterious stresses produce a deleterious phenotype when present together (Beijersbergen et al., 2017; Setton et al., 2021; van Leeuwen et al., 2017). In some cases of synthetic lethality, the resulting death is due to the pair being involved in the same pathway. Alternatively, it remains possible that the observed *pxl1*Δ-copper lethality is non-specific. To address these possibilities, we examined the effect of expressing a functionally independent pro-survival sequence namely human 14-3-3 in *pxl1*Δ yeast. As shown in Fig 7B, 14-3-3, like CSRP3, reverses the effects of copper on *pxl1*Δ cells. Both 14-3-3 and CSRP3 expression not only serve to promote the growth of copper challenged *pxl1*Δ cells, they also serve to prevent cell death (Fig 7C).

To further address the specificity of the *pxl1*Δ-copper lethality, we examined the ability of *pxl1*Δ cells to grow with SDS at a concentration that mildly inhibits the growth of WT cells. Here we show that the addition of 0.0075% SDS to cells lacking *pxl1*Δ prevents cell growth while wild type cells are only moderately inhibited (Fig 7D). Given that SDS and copper are chemically unrelated further supports the concept that the observed synthetic lethality of combined *pxl1*Δ-copper is likely non-specific.

3.1.8 Analysis of stress responses in CSRP3 mutants

Finally, we asked the question does CSRP3 require the function of its own LIM domains to mediate a pro-survival effect in yeast. To this end, using site directed mutagenesis, point mutations were individually introduced into each LIM domain of CSRP3. The residues that coordinate zin ions are likely to be structurally important. In corroboration, a review of known mutations in CSRP3-related cardiomyopathy revealed a cysteine to guanine change (C58G) that coincides with a zinc coordinating residue in LIM1 (Fig 8). Yeast were then transformed with plasmid containing this mutant and is referred to as $CSRP3_{C58G}$. Additionally, to assess the requirement of the second LIM domain, mutants containing a truncated version of CSRP3 lacking LIM2 were generated by prematurely inducing a stop codon before the coding region of the second LIM domain. Using growth and viability assays, the ability of these variants to protect against copper-mediated RCD was measured. In spot assays, the growth of yeast expressing $CSRP3_{C58G}$ and $CSRP3_{E118X}$ grew more than control. In comparison to yeast expressing CSRP3, the growth of the mutants was comparable. When measuring the viability of yeast, neither mutations to CSRP3 significantly affected its ability to protect against exogenous excess copper stress when compared to the native CSRP3 (Fig 8). Taken together, these data suggest that the pro-survival characteristics conferred to yeast overexpressing CSRP3 do not require LIM domains; neither those of CSRP3 nor that of endogenous LIM containing genes.

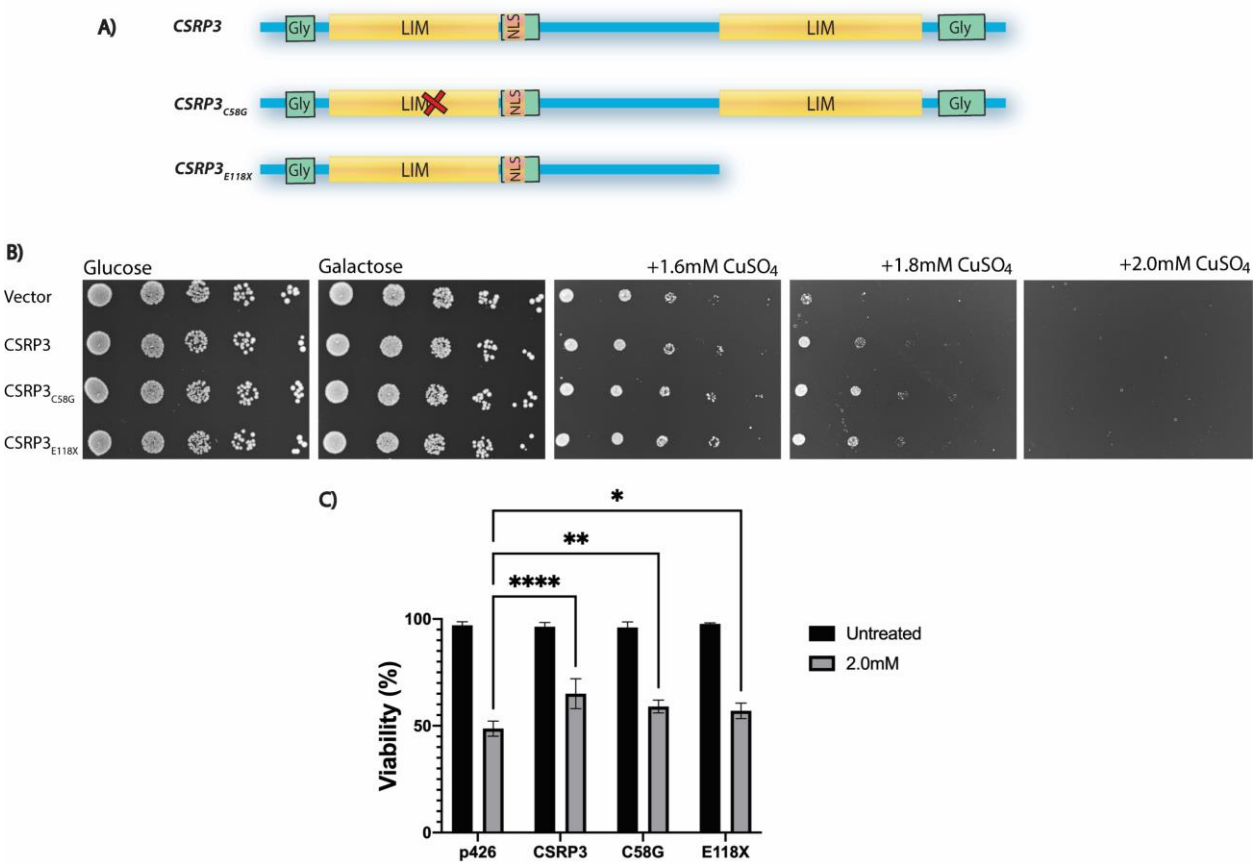

Figure 8. ***Analysis of copper sensitivity of CSRP3 mutants reveals that LIM domains are not required to protect against copper stress.*** ***A)*** *Linear representation of CSRP3 protein structure and mutants.* ***B)*** *Wild type yeast transformants harbouring plasmids expressing different variants of CSRP3 under the control of the* GAL1 *promoter were analyzed using the spot assay. Aliquots of serial dilutions of cultures of the different transformants were spotted onto YNB nutrient agar plates containing Glucose, Galactose, or Galactose and copper sulphate ($CuSO_4$). The plates were incubated for 3 days and then photographed.* ***C)*** *Growing cultures of each yeast strain were treated with or without the indicated level of copper stress for 18h. Cell samples were then microscopically examined using trypan blue. Viability is represented as the proportion of trypan blue negative cells.*

3.2 Investigating the Toxicity of Metal Salts in Yeast using Pro-Survival Sequences

Our lab has successfully identified several human and yeast pro-survival sequences by direct screening of mammalian cDNA expression libraries in yeast cells undergoing RCD due to ectopic BAX expression (Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012; Eid et al., 2016; Eid et al., 2014; Horowitz et al., 2013; Jones et al., 2015; Sheibani et al., 2015; Zhou et al., 2020). Many of the clones identified have since been confirmed to be pro-survival against BAX and copper stress, but we have not fully explored how these different sequences affect the regulation of different PCD subroutines. One way to test this is to screen pro-survival sequences against different stresses known to induce RCD.

As previously mentioned, the study of metal toxicity is of interest for both our understanding of human and cellular health as they pose great risks in high doses. Indeed, the mechanisms by which many of these metals mediate their toxicity is not fully understood. Nonetheless, it is reasonable to assume that the mechanism of action by which certain agents operate within a cell relates to their toxicity. Thus, it is possible that the ability of pro-survival sequences to inhibit stress-mediated cell death may intersect with these processes. Humanized yeast models undergoing RCD offer a system by which mechanistic details can be teased out. To this end, we report on findings of both CSRP3 and 14-3-3 expression against different stresses and their resulting phenotypes.

3.2.1 Heterologous expression of pro-survival sequences protects against copper-mediated RCD and enhances iron-mediated RCD

The use of iron as a stress is of high interest since like copper, it is an essential element but also toxic at high levels (Winterbourn, 1995). Iron is also known to increase the production of ROS via Fenton reactions (Winterbourn, 1995). Thus, iron toxicity may lead to DNA damage and other typical markers of apoptosis and RCD. Using spot assays, cells heterologously expressing previously identified pro-survival sequences 14-3-3 and CSRP3 show the same growth pattern as control in the absence of stress (Fig 9A,C). When exposed to RCD-inducing levels of copper, cells expressing either sequence similarly demonstrated both increased growth on nutrient agar as well as significantly higher viability when compared to control (Fig 9A,C). This is consistent with our previous findings of novel pro-survival sequences identified from screening cDNA expression libraries in BAX-expressing yeast inhibit stress-mediated RCD such as with copper. For example, cells expressing human thyroid cancer 1 (TC-1) are also able to grow more than control in the presence of copper stress (Jones et al., 2015). Interestingly, when challenged with excess exogenous iron, the growth of yeast expressing pro-survival sequences were noticeably less than that of control (Fig 9A,C). This difference in phenotype against two different cell death-inducing stresses suggests that the expression of pro-survival sequences may induce a RCD subroutine where cell death is enhanced by their expression. The fact that the response to iron is similar between 14-3-3- and CSRP3 also suggests that this response is not specific and is in fact also observable with other previously identified pro-survival sequences (Clapp, Portt, Khoury, Sheibani, Norman, et al., 2012; Eid et al., 2016; Eid et al., 2014; Horowitz et al., 2013; Jones et al., 2015; Sheibani et al., 2015; Zhou et al., 2020). The effects of exogenous iron stress on growth is also paralleled by viability in yeast (Fig 9B). The viability of cells grown in the presence of iron were directly using by microscopically examining cells stained with the vital dye

trypan blue. For all strains, in the absence of stress, viability was observed to be above 98% (Fig 9). In the presence of copper, 14-3-3 expressing cells were observed to have a significantly higher percentage of viable cells compared to control (Fig 9). In contrast, cells expressing 14-3-3 had a significantly lower viability compared to control (Fig 9B). These data suggest the existence of an RCD subroutine with iron that unlike copper, is paradoxically enhanced by the expression of pro-survival sequences.

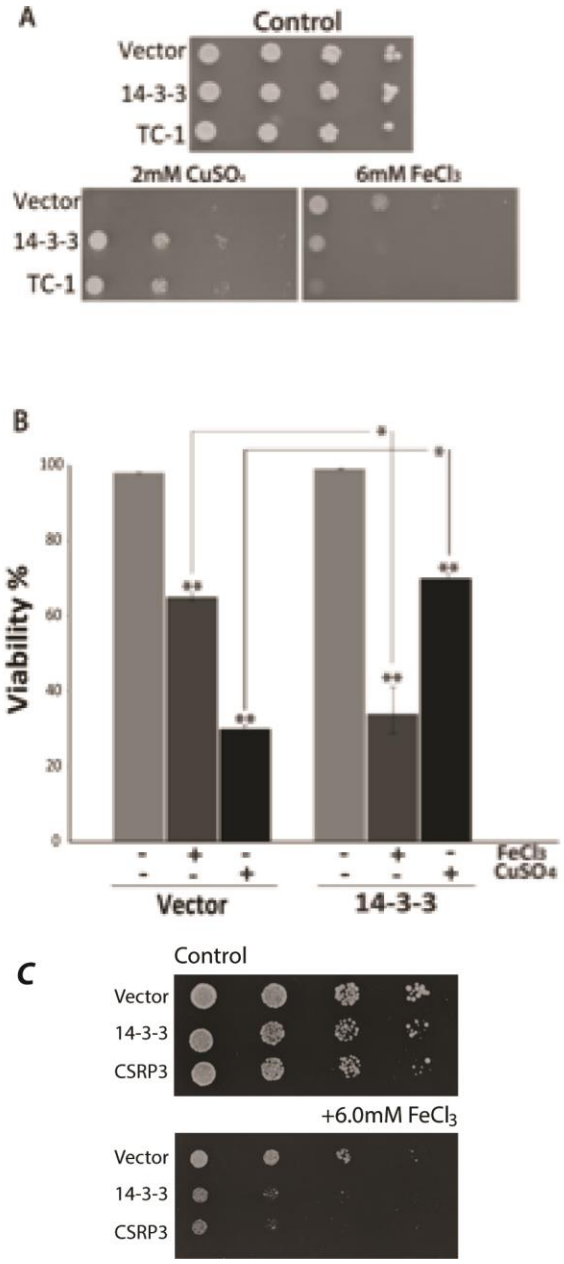

Figure 9. Effects of iron stress on yeast. A) *Spot assays depicting the growth of WT yeast harboring plasmids containing previously identified PCS sequences under the control of* GAL1 *promoter under normal, excess copper and iron conditions. Serially diluted aliquots of yeast were grown for 72h on nutrient agar and subsequently photographed.* ***B)*** *Samples of yeast cultures grown in media containing excess copper or iron were measured for viability. Viability was*

determined microscopically using the vital dye trypan blue and expressed as the percentage of trypan blue negative cells. ***C)*** *Spot assays of WT yeast transformed with the novel PCS sequence CSRP3 were performed. Serially diluted aliquots were grown on nutrient agar in the presence and absence of excess iron.*

3.2.2 Effects of copper and iron stress on yeast RCD mutants

To further address the differences between yeast stress responses to excess copper and iron when expressing different pro-survival sequences, yeast mutants defective in RCD were used (Fig 10). For example, the yeast metacaspase YCA1 is involved in acetic acid-mediated cell death but not copper (Liang & Zhou, 2007; Madeo et al., 2002). Thus, mutants defective in key RCD regulators such as YCA1, YBH3 (BH3 containing BAX-like protein), and ATG1 (autophagy-defective) were used to gain insight into how iron and copper stress differed in yeast. Each mutant was transformed with empty vector or those containing 14-3-3 and grown in the presence of copper and iron (Fig 10A). As shown previously, the growth of WT yeast is inhibited by exogenous copper and iron. Similarly, the expression of 14-3-3 protected yeast against the deleterious effects of copper whilst enhancing the effects of iron (Fig 10A). In yeast RCD mutants, the absence of key RCD regulators had no discernable effect on the growth of yeast both in the absence or presence of stress (Fig 10A). Thus, YCA1, YBH3 and ATG1 are not likely to be involved in mediating the effects of both copper and iron in wild type and pro-survival expressing cells.

In yeast, the vacuole plays a critical role in the metabolism of iron. Indeed, many yeast mutants defective in vacuolar function have increased sensitivity to iron stress (Eid et al., 2016; Li

et al., 2001; Szczypka et al., 1997). Here, we asked the question what the effect of defective vacuolar function is in yeast expressing empty vector or 14-3-3 under copper and iron stress.

In spot assays, the growth of both of wild type and mutant yeast expressing either empty plasmid or 14-3-3 had no discernable differences in growth in the absence of stress (Fig 10B). In the presence of sublethal copper stress, yeast lacking VMA3 experienced growth inhibition and grew less than wild type but could be rescued by the expression of 14-3-3. Under iron stress, growth of wild type yeast, as previously shown was inhibited by the addition of 6mM $FeCl_3$ and even more if 14-3-3 was expressed. Interestingly, in yeast lacking VMA3, while control cells were sensitive to sublethal iron, they could surprisingly be rescued by the expression of 14-3-3 (Fig 10B). These data suggest that the response to iron in WT and VMA3-lacking yeast differ and that in iron mediated RCD, the vacuole is involved.

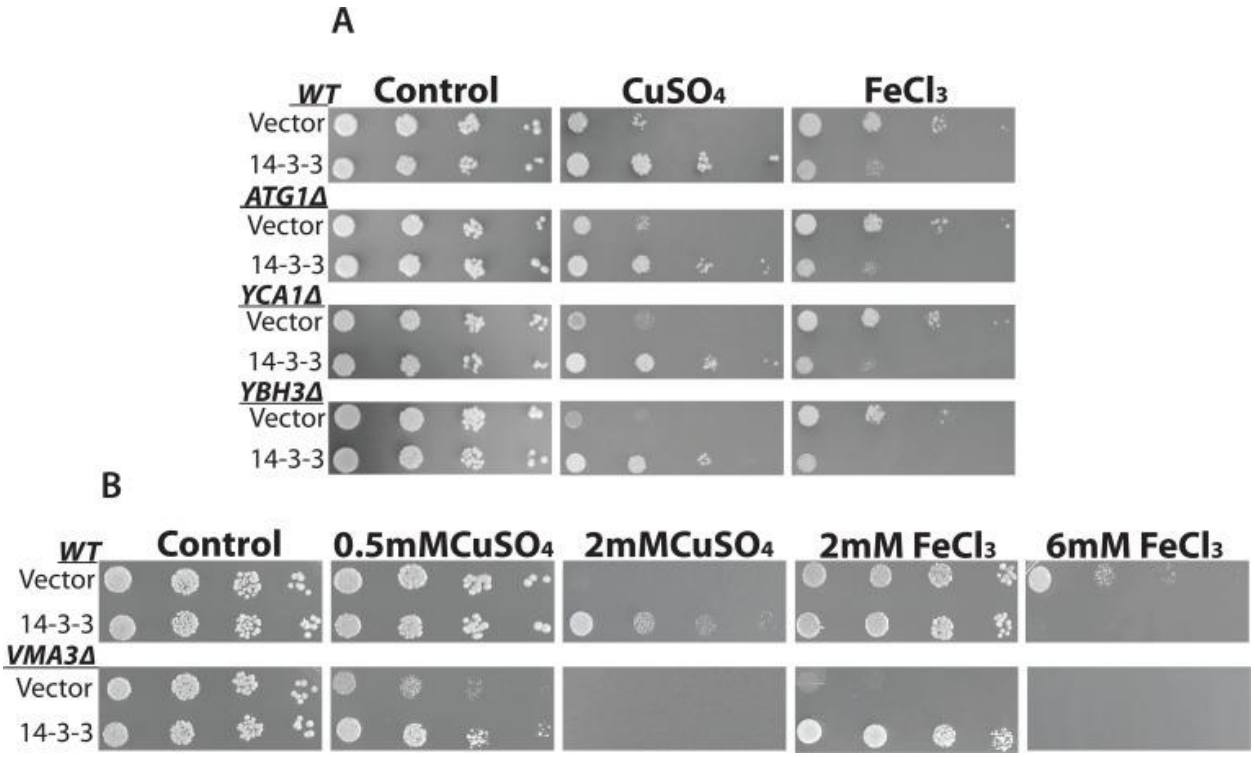

Figure 10. Effect of iron and copper stress on yeast RCD mutants. A) *Spot assays of WT and mutant yeast were performed on nutrient agar in the presence and absence of excess copper or iron. Growth was measured and photographed after 72h.* ***B)*** *Spot assays of WT and mutant yeast lacking VMA3 transformed with empty vector or 14-3-3 were performed in the presence of varying doses of excess copper and iron stress.*

3.2.3 Copper conditioning prevents iron-mediated growth inhibition

Different RCD pathways often share cross talk between one another (Portt et al., 2011). As previously shown, the response to copper and iron stress differed between wild type and vma3Δ yeast expressing a pro-survival sequences. We thus asked the question whether there is cross-talk between copper and iron-responsive RCD pathways in yeast. To this end, spot assays were performed using yeast cells expressing empty vector or 14-3-3 (Fig 11). Yeast cells grown in the presence of 1.6mM $CuSO_4$ showed no discernable growth differences with their respective controls indicating that this is a sub-lethal level of copper (Fig 11). In the presence of iron stress, consistent with previous findings, the growth of yeast was inhibited and this growth inhibition was enhanced by the expression of 14-3-3 (Fig 11). Surprisingly, yeast pre-conditioned with sublethal levels of copper and subsequently challenged with lethal levels of iron stress grew more when compared to iron alone (Fig 11). Taken together, these data suggest that the pre-treatment of yeast with sub-lethal levels of copper mitigates the deleterious effects of iron, lending further support to the possibility of crosstalk between the two stress-responsive pathways.

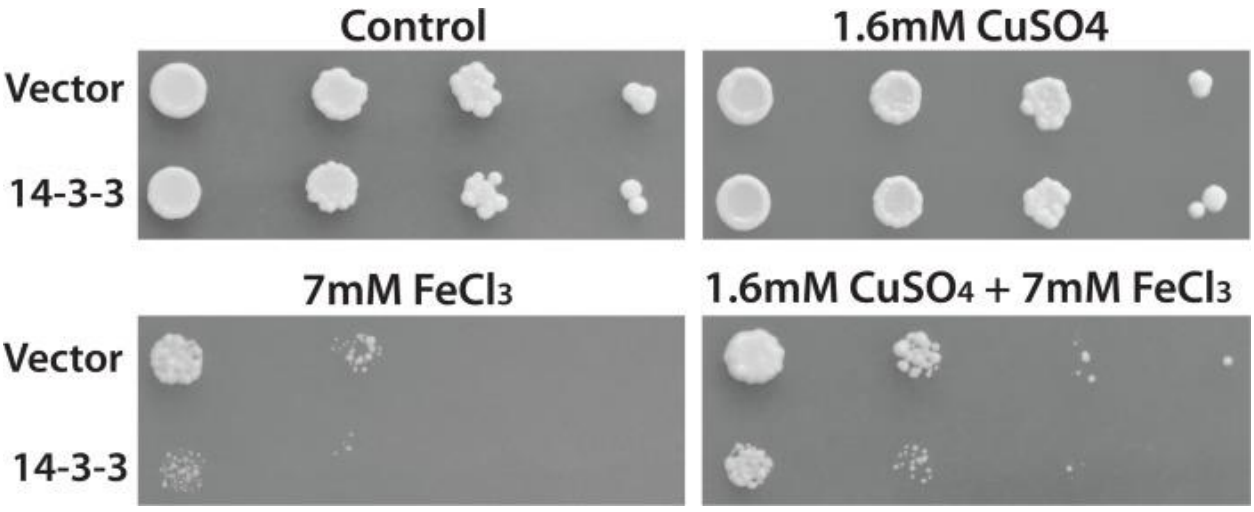

Figure 11. Pre-conditioning with sub-lethal levels of copper reduces the deleterious effects of excess iron stress. *Spot assays were performed with WT and vma3*Δ yeast harboring empty plasmids or that containing 14-3-3 on nutrient agar containing copper and iron stress. Cultures of yeast were also pre-treated with copper before being spotted onto nutrient agar containing lethal levels of iron. Growth was observed and photographed after 72h incubation.

3.2.4 Re-examining the effects of exogenously added iron experiments with yeast

While it is clear in both liquid and spot assays that the addition of excess iron has deleterious effects on both yeast growth and viability, one of the major challenges of studying iron stress in yeast is the immediate formation of white precipitate in liquid media. While this has been a long-recognized problem in some fields, it is surprisingly largely under reported in toxicity studies of iron (Dalton et al., 1983; Eid, Arab, et al., 2017).

3.2.4.1 Iron-mediated growth inhibition and cell death is preceded by the formation of precipitate in yeast media

In our previous published studies, the addition of iron to yeast growth media immediately led to the formation of a precipitate independent of the presence of cells (Eid, Zhou, et al., 2017). This accumulation of a precipitate proceeded in a dose dependent manner much like the dose-dependent inhibition of yeast growth in the presence of iron stress.

To further understand iron toxicity in laboratory conditions, we sought to investigate the relationship between the formation of precipitate and the inhibition of yeast growth and viability. We therefore asked the question: is iron's toxicity in standard cell culture media the result of precipitating an essential component of media. Yeast YNB media was inoculated with increasing amounts of both exogenous $FeCl_3$, $FeSO_4$ and $CuSO_4$ as a control (Fig 12A). In addition, spot assays using the same concentrations of these metal salts were performed to compare their effect on yeast growth. As with previous assays, spot assays of freshly grown yeast cultures with empty vector or with 14-3-3 were performed with and without increasing concentrations of $FeCl_3$, $FeSO_4$ or $CuSO_4$. As expected, the inhibition of yeast growth was observed at 7mM $FeCl_3$. Consistent with previous findings, the inhibition of yeast growth was enhanced by the expression of pro-survival sequences including 14-3-3.

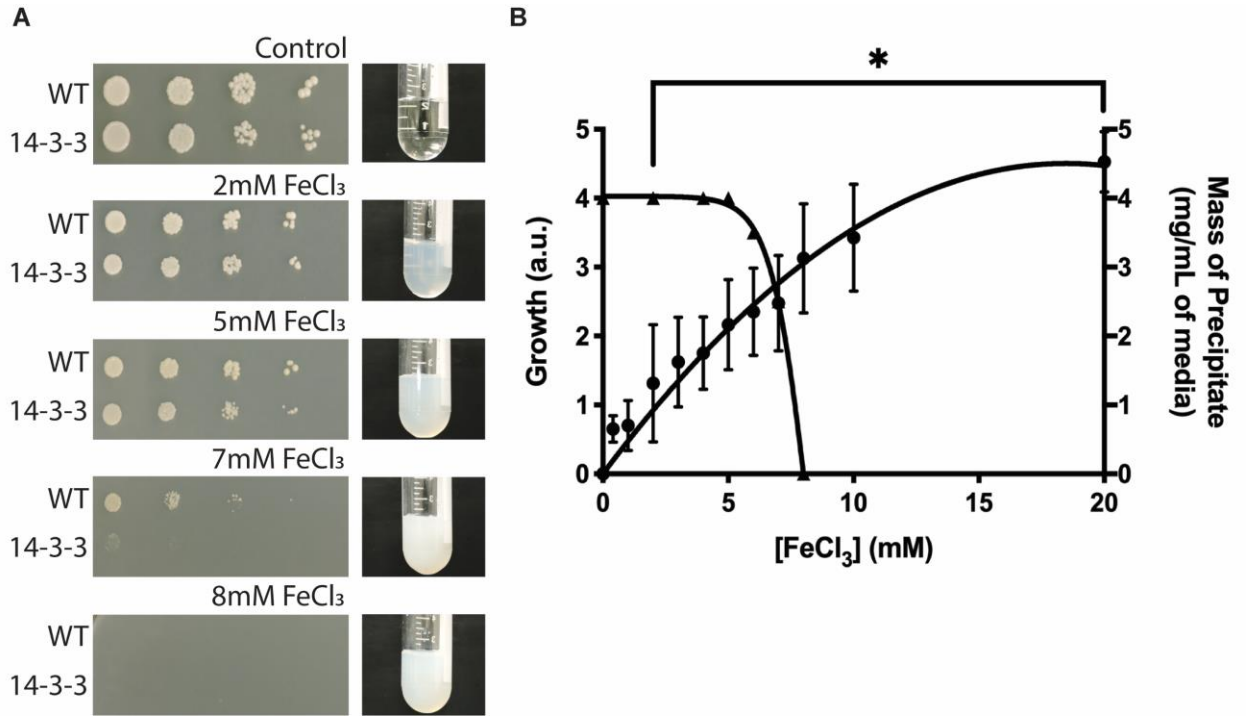

Figure 12. The precipitation of iron in yeast media occurs in a cell independent and dose dependent manner. A) *Visual comparison of the formation of precipitate following the addition of increasing amounts of iron into liquid yeast media against the growth of WT and 14-3-3 expressing yeast under the same concentration.* ***B)*** *Quantitative analysis of the amount of precipitate formed compared to the amount of growth inhibition in spot assays.*

As we previously reported, the addition of iron salt at a concentration as low as 0.4mM to minimal yeast growth media led to the accumulation of an uncharacterized precipitate in the media (Eid, Zhou, et al., 2017). We thus hypothesized that exogenous iron reacted with some component in the media which caused the formation of a precipitate. We further hypothesized that the formation of the precipitate drove the toxic effects of iron in media. To investigate this possibility, we firstly measured the effects of adding increasing amount of iron in yeast media alone (Fig 12).

As shown, normal YNB yeast media is a clear solution but the addition of iron to media causes the formation of a precipitate that changes the appearance of media to cloudy.

To understand the relationship between iron-induced precipitation in media and its deleterious effects, a dose-dependent precipitation experiment was performed. Using YNB media alone as a control, different increasing concentrations of iron were incubated in media without cells for 24 hours and photographed to qualitatively document the precipitation (Fig 12). In parallel, spot assays using the same concentrations were performed using WT yeast and yeast expressing 14-3-3. At concentrations between 2mM and 5mM, it was observed that the addition of iron caused the formation of precipitate but at those concentrations, little or no effect was noticeable on the growth of yeast in spot assays. At higher concentrations of 7-8mM, the growth of yeast is greatly inhibited. To quantitatively measure the amount of precipitation in media, solutions containing yeast media and iron were centrifuged and the resulting precipitated pellet was weighed. The mass of precipitate accumulated increased correspondingly with iron concentration from 0.4-20mM (Fig 12). Half-maximal accumulation was observed at around 5mM. The accumulation of precipitate was eventually observed to plateau at a maximum of 4.5mg. Taken together, the kinetics of both processes seen in these data supports the notion that the formation and accumulation of a precipitate is responsible for irons toxic effects in yeast media. Interestingly, this phenomenon of nutrient depletion is commonly documented for plant cell cultures but has yet to be described for yeast and mammalian cell cultures (Sanchez-Rodriguez et al., 2014).

3.2.4.2 The effect of oxidation state on iron toxicity

Iron exists in multiple oxidation states and can stably interconvert between ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms. These different oxidation states also have different properties in terms of solublity and differential biological functions as either electron acceptor or donor. We therefore asked if the oxidation state of iron affects its toxicity in yeast. To test this, the same experiments were performed with ferrous iron. Control cells harboring empty vector grew normally at 1mM $FeSo_4$ and growth inhibition was first noticeable at 50mM. Complete growth inhibition was achieved around 100mM. As a positive control, cells expressing 14-3-3β/α were similarly observed to grow less than control in the presence of iron. Thus, these results demonstrate that ferrous iron, like ferric iron, is also a dose-dependent inhibitor of yeast growth.

Interestingly, it takes much more ferrous iron (100mM) than ferric (~6-8mM) to significantly inhibit growth. We therefore sought to investigate if ferrous iron also caused the formation of precipitate as observed with ferric iron. Using the same experimental protocol, media containing no additional ferrous iron was compared against solutions containing increasing concentrations of $FeSO_4$. As observed with ferric iron, the addition of ferrous iron to media as low as 1mM caused the formation of a precipitate in a dose dependent manner (Fig 13). Similarly, the formation of precipitate occurred at concentrations that precede the minimum concentration required to inhibit the growth of yeast in plates. Thus, like ferric iron, ferrous iron causes precipitation in media at concentrations lower than that needed for growth inhibition (Fig 13).

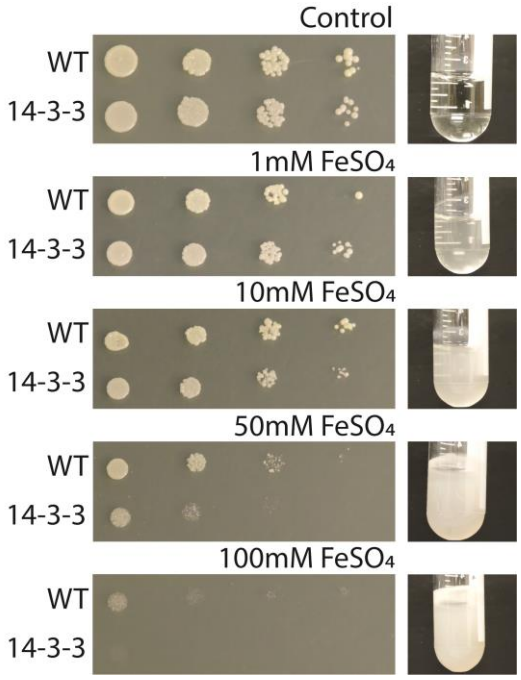

Figure 13. Ferrous iron is a dose dependent inhibitor of yeast growth and precipitates in yeast media. *Visual comparison of the formation of precipitate following the addition of increasing amounts of iron into liquid yeast media against the growth of WT and 14-3-3 expressing yeast under the same concentration.*

3.2.4.3 Copper toxicity does not require precipitation

On the other hand, copper, like iron, is a transition metal and essential micronutrient that is toxic to cells and capable of inducing PCD in both mammalian and yeast cells. Based on this, it was of interest to determine how copper toxicity differed from iron in terms of the formation of a precipitate. Using the same experimental protocols as above, the growth of yeast cells in the presence of copper on spot assays was compared to qualitative observations of copper in media

alone (Fig 14). As expected, the growth of control cells in the presence of copper was inhibited at 2mM $CuSO_4$ and could be protected by the expression of 14-3-3.

To determine if precipitation occurs with excess copper, increasing amounts of $CuSO_4$ were added to YNB media at varying increasing concentrations (Fig 14). Up to a concentration of 50mM $CuSO_4$, no precipitation was detected suggesting that unlike iron where opacity was immediately observable even at low concentrations, copper does not involve the formation of a precipitate of which is not necessary for its toxicity.

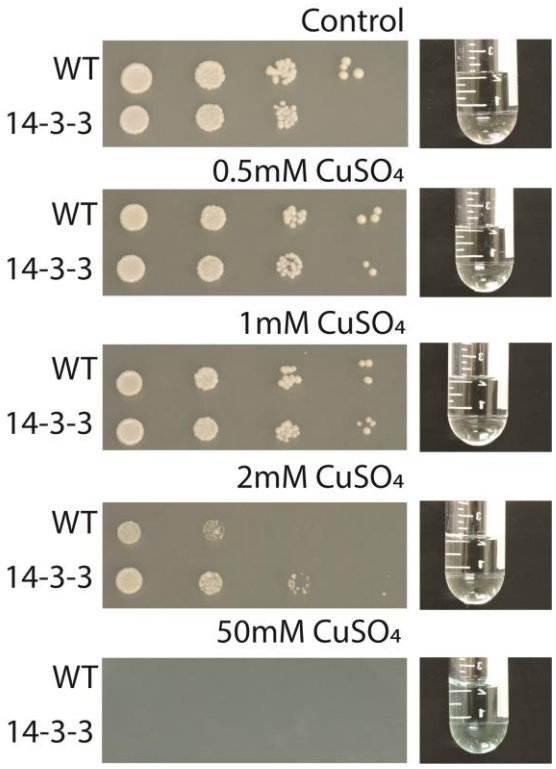

Figure 14. Toxicity of excess copper does not involve precipitation in media. *Visual comparison of the formation of precipitate following the addition of increasing amounts of iron into liquid yeast media against the growth of WT and 14-3-3 expressing yeast under the same concentration.*

3.2.4.4 The addition of excess phosphate reverses iron-mediated growth inhibition

YNB is a defined media that contains all trace elements and several salts that are required for yeast growth (Fig 15). Among all ingredients in YNB, phosphate is the most likely component of YNB capable of forming a precipitate with iron (Fig 15). It is present at sufficiently high concentrations to stoichiometrically account for the mass of precipitate obtained. In support of this, it is known that phosphate is well known to be insoluble with iron. Furthermore, iron is the only anion in YNB that is insoluble with phosphate (Fig 15). Finally, the depletion of phosphate is a known stress that induces cell death (Almeida et al., 1992). Taken together, we hypothesized that the addition of iron in YNB media and subsequent formation of a precipitate involves phosphate. Furthermore, if true, the addition of excess phosphate should reverse the effects of iron-mediated growth inhibition in a dose dependent manner.

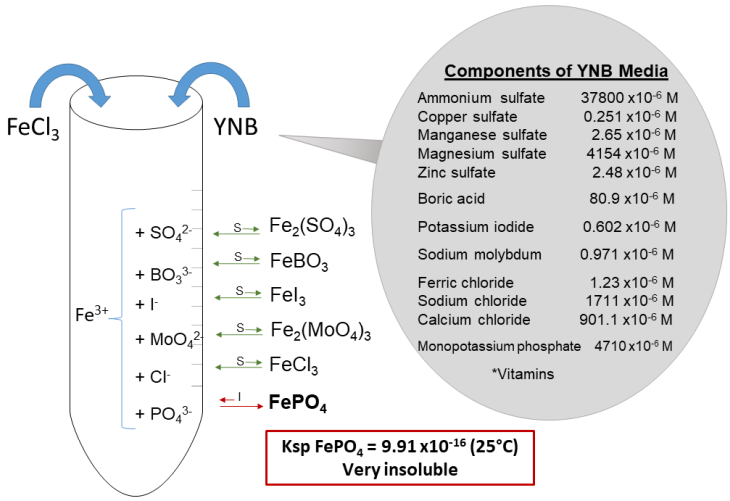

Figure 15. Composition of YNB media. *Schematic representation of the chemical composition of the salts presents in yeast YNB media. The solubility of ferric (Fe^{+3}) with the different salts are shown as equations and the equilibrium constants of Fe^{+3} and PO_4^{-3} are also shown.*

To test this possibility, spot assays were used to measure the effects of excess exogenous phosphate in media in the presence and absence of iron (Fig 16). As previously shown, the addition of iron causes the inhibition of yeast growth around 7-8mM $FeCl_3$ and this effect is enhanced when cells express pro-survival sequences including 14-3-3 and CSRP3. In the presence of excess iron, the additional presence of excess phosphate reverses the growth inhibitory effects in plates (Fig 16). A noticeable effect is observed when phosphate levels are doubled (2X, or 2g/L) over the basal levels of phosphate (1X, or 1g/L) found in standard YNB media. Further increases in growth were subsequently observed with higher concentrations of excess phosphate. Surprisingly, the addition of excess phosphate is even capable of reversing the iron-sensitive phenotype of 14-3-3 expressing cells. Thus, these results support the claim that the growth inhibitory and death inducing effects of iron are not specific to iron but likely driven by the depletion of an essential component of media.

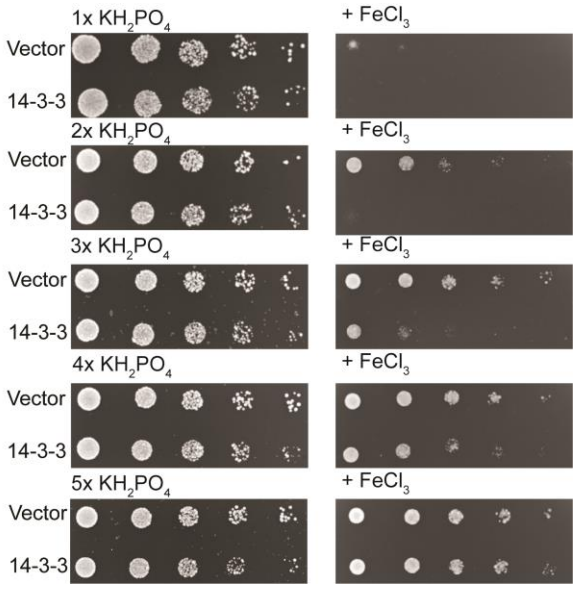

Figure 16. Effect of adding increasing concentrations of exogenous phosphate with excess iron. *Spot assays were performed using WT yeast harboring empty plasmid or expressing 14-3-3. Yeast were grown on nutrient agar containing increasing amounts of equivalents of phosphate in the*

absence and presence of deleterious levels of excess iron. Plates were incubated for 72h and subsequently imaged.

3.2.5 CSRP3 protects against Lithium stress and requires MCK1

Previously in this thesis, we characterized CSRP3 as a novel pro-survival sequence capable of protecting against RCD-inducing stresses including ectopic BAX expression and excess exogenous copper. Despite it being unclear as to exactly how it mediated this effect, we were nonetheless interested in its protective range. To further understand the scope of CSRP3's protection against stress in yeast, it was screened against several different RCD-inducing compounds including other metal salts (Fig 17). Interestingly, in the presence of lithium stress, the expression of CSRP3 in yeast helped to resist its growth inhibitory effects.

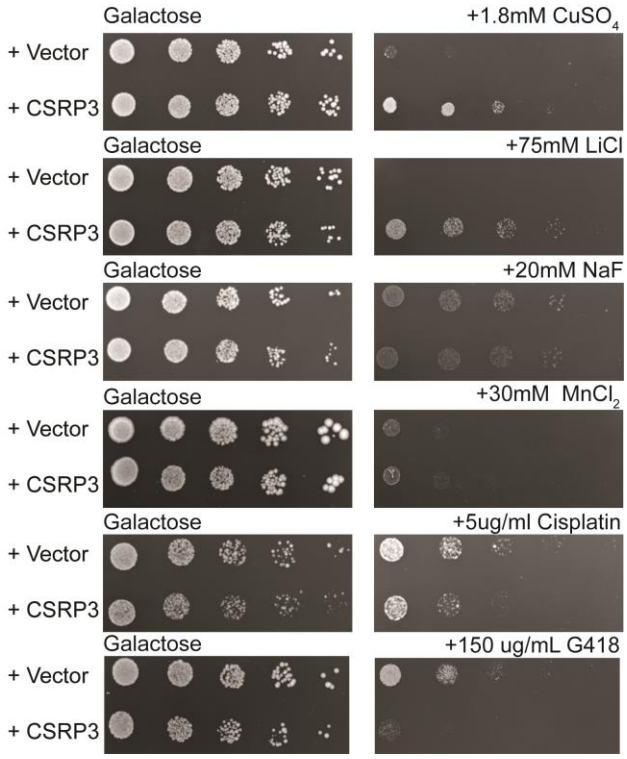

Figure 17. The response of CSRP3-expressing yeast under various RCD-inducing stress. *Spot assays were performed using WT and CSRP3-expressing yeast in the presence and absence of different-RCD inducing stresses. Yeast were incubated for and imaged after 72h.*

While lithium is not an essential element, it is of interest as it is routinely prescribed and used as a therapeutic agent for the treatment of bipolar disorder. Even after almost a century of clinical use, the exact mechanism by which lithium acts inside cells remains unclear. Despite its therapeutic potential, the use of lithium is complicated by its potential toxicity due to its narrow therapeutic window (Amdisen, 1988). Thus, many studies have aimed to explore the mechanisms by which lithium is toxic to cells. Several have proposed that the effects of lithium involve the regulation of endogenous inositol synthesis as well as components of GSK3 signalling in both mammalian and yeast cell models (Gould et al., 2004; Lopez et al., 1999; O'Brien & Klein, 2009; Vaden et al., 2001). To this end, we further assessed lithium toxicity in yeast. Using spot assays, yeast expressing either empty vector, 14-3-3 or CSRP3 were grown in both the presence and absence of increasing amounts of lithium stress (Fig 18). As expected, yeast harboring either empty vector, or expressing 14-3-3 or CSRP3 showed no discernable differences in growth when grown on galactose inducible media. In the presence of exogenous lithium stress, control cells demonstrated clear growth inhibition at 60mM LiCl (Fig 18). Consistent with our previous screen, the expression of CSRP3 protected yeast against growth inhibition. Interestingly, the expression for 14-3-3 also protected against the deleterious effects of excess lithium (Fig 18). This further supports the characterization of the expression of CSRP3 alongside 14-3-3 as a general pro-survival tool. To examine the effects of lithium toxicity on yeast viability, as with copper, cells grown in culture were observed microscopically using the vital dye Trypan Blue. In the absence

of stress, WT and CSRP3-expressing yeast had no discernible difference in viability. In the presence of copper or lithium, the viability of cells expressing CSRP3 or 14-3-3 were significantly higher than that of control indicating that they also protected against the death-inducing effects of excess lithium stress.

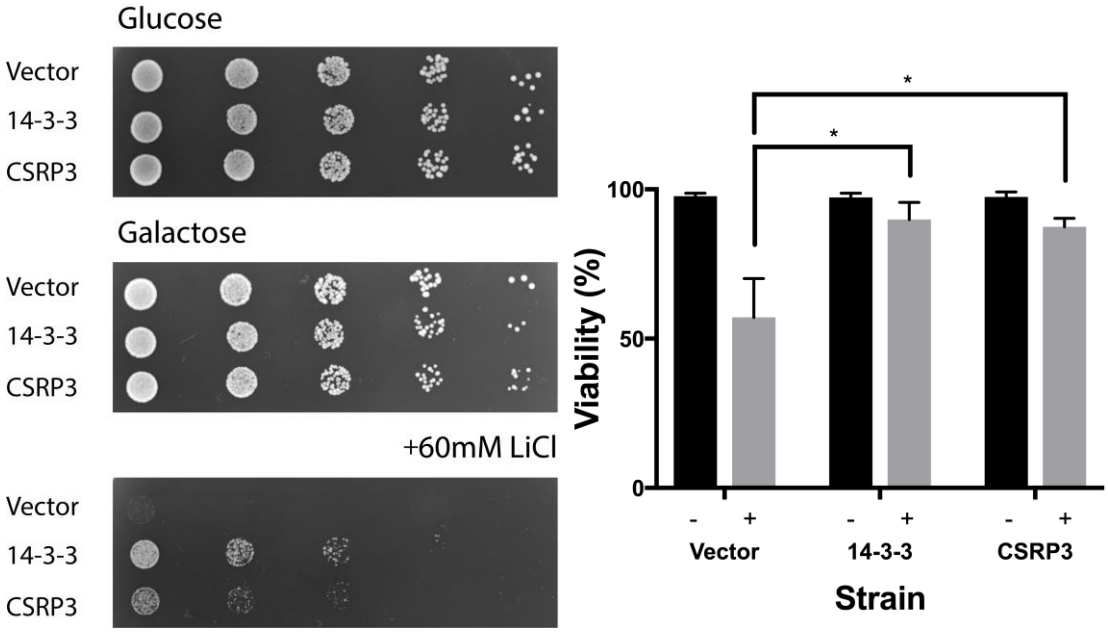

Figure 18. CSRP3 expression protects against lithium stress. *Spot assays were performed with WT yeast harboring empty vector or transformed with 14-3-3 or CSRP3. Serially diluted aliqots of growing yeast were spotted onto nutrient agar containing the indicated conditions and grown for 72h at 30C. Viability assays were also performed using the vital dye trypan blue. Viability is expressed as the percent of trypan blue negative cells. * indicates statistical significance.*

Based on these data, we then asked what the effect of lithium stress and the heterologous expression of general pro-survival sequences such as CSRP3 was on yeast mutants defective for

regulators of de novo inositol synthesis and GSK3 signalling. Using spot assays, the growth of wild type and yeast knockout strains defective for endogenous inositol regulators INO1, INO2 (Fig 19) as well as orthologous GSK3 signalling regulators MCK1, YGK3, MDS1, MRK1 (Fig 20) was measured in the presence and absence of excess lithium. In the absence of stress, all strains had no discernible differences in growth indicating that the loss of any single gene did not have a deleterious effect (Fig 19, 20). In the presence of lithium stress, the loss of either INO1 nor INO2 did not affect the ability of CSRP3 to protect against lithium (Fig 19). This suggests that CSRP3's protection against lithium toxicity in yeast does not require the endogenous regulation of inositol synthesis. In the absence of endogenous GSK3 orthologues in yeast, CSRP3 could protect against lithium stress in yeast lacking YGK3, MDS1 and MRK1. Surprisingly, the loss of MCK1 entirely abrogated CSRP3's ability to protect against the effects of lithium in spot assays (Fig 20). Taken together, this finding suggests that CSRP3 requires MCK1 in yeast to protect against lithium stress, lending further credence to GSK3 signalling as the mechanism of action driving lithium toxicity. It would be interesting in future experiments to see if CSRP3 interacts with MCK1.

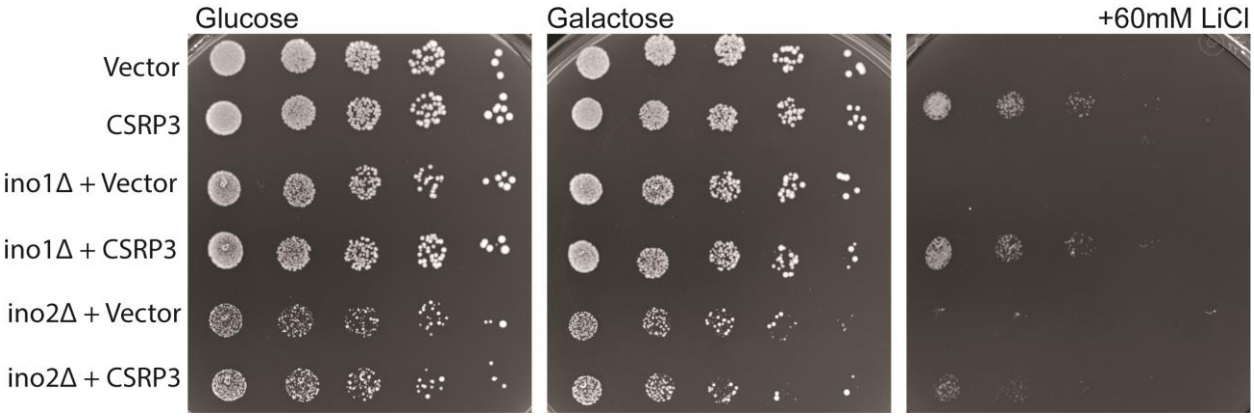

Figure 19. Analysis of the stress response of yeast knockouts defective for endogenous inositol synthesis genes to excess lithium. *Spot assays were performed with WT yeast and yeast lacking*

either INO1 or INO2. Yeast strains were transformed with both empty vector as a control and CSRP3. Yeast were grown on nutrient agar for 72h and subsequently imaged.

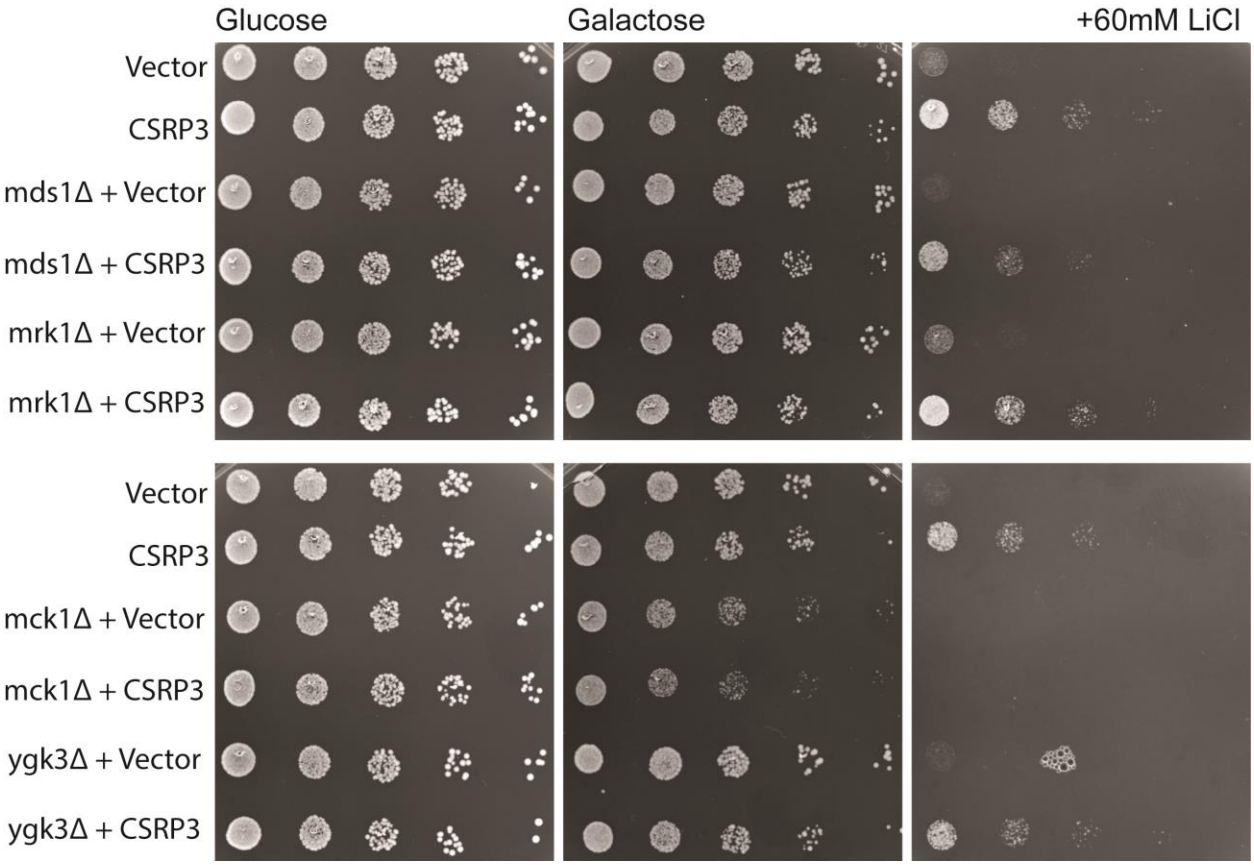

Figure 20. Analysis of the stress response of yeast knockouts defective for endogenous GSK3 signalling orthologues to excess lithium. *Spot assays were performed with WT yeast and yeast lacking either MDS1, MRK1, MCK1 or YGK3. Yeast strains were transformed with both empty vector as a control and CSRP3. Yeast were grown on nutrient agar for 72h and subsequently imaged.*

3.3 Stress intensity does not uniformly predict stress response in yeast

In experiments with metals salts acting as exogenous stressors, it was observed that the amount of their growth inhibitory and cell death inducing effects on growing yeast could be modulated by the overexpression of a pro-survival sequence. The fact that stress responses can be affected by the expression of genes suggest that the paradigm in which stress intensity dictates stress response is a malleable concept, especially when concerning the threshold at which cells undergo RCD. We furthered our inquiry into this model of stress intensity vs response by verifying whether extreme levels of stress correlated with typical expected stress responses such as programmed cell death as well as necrosis. Since yeast harbor cell walls, the typical "blown up" appearance of necrotic mammalian cells are unobservable when working with yeast. Nonetheless, in the presence of extreme or necrotic levels of stress, we expected that there would be no detectable viable cells, akin to boiling cells at 100C. Furthermore, as previously discussed, necrosis occurs on a relatively short time scale (i.e. 30 seconds at 100C results in complete cell death in cell cultures). Thus, as a preliminary criterion, we sought to measure at what intensity (magnitude and time) complete loss of viability occurred at in yeast under different stress. With this in mind, yeast were subjected to different concentrations of different stressors known to induce cell death.

3.3.1 Stress responses to ethanol

Ethanol is a widely used sanitizing agent and relatedly, a potent and commonly used cell-death inducing stress in laboratory conditions. In yeast, the use of ethanol has also been shown to induce RCD with morphological similarities to mammalian apoptosis, further lending credence to its use as a general RCD inducing agent (Kitagaki et al., 2007). As a start, ethanol stress was used to test the relationship between stress intensity and stress response in yeast. In response to increasing concentrations of ethanol stress, the viability of growing cultures of yeast began to decrease at concentrations beyond 15% ethanol after 18h incubation. Viability continued to decrease sharply with increasing concentrations of ethanol (Figure 21). The minimum concentration required to induce complete cell death in yeast is approximately 50%. This is consistent with practical application of ethanol in the real world as most ethanol based agents are recommended to contain at least 60% EtOH.

Yeast cultures respond differently to some stress depending on their growing status (Elliott & Futcher, 1993). To this end, it is thought that stationary yeast cultures are relatively more stress resistant than growing ones although whether this relationship is causative remains unclear (Elliott & Futcher, 1993). For example, rapidly growing yeast are sensitive to heat shocks while stationary cultures are highly resistance (Elliott & Futcher, 1993). To assess the influence of growth status on stress response to ethanol, both stationary yeast were similarly subjected to increasing amounts of EtOH stress (Figure 21). While the treatment of stationary yeast cultures with increasing concentrations of ethanol for 18h also resulted in a dose-dependent decrease in cell viability no distinct differences in viability were observable between stationary and growing yeast cultures (Fig 21).

Necrosis is a rapidly occurring unregulated death. Typical RCD programs in mammalian cells such as apoptosis can take as little as 2-3 hours or as long as 24h to complete (Saraste &

Pulkki, 2000). Thus, treatment of yeast with stress for 18h may be an inappropriate time frame to accurately capture necrotic events and distinguish them from RCD. To refine our inquiry into stress intensity vs response, the above experiments were repeated using 5 min and 2h exposures to increasing concentrations of ethanol. Interestingly, while no discernable differences in viability were observed between stationary and growing yeast, the duration of exposure resulted in a stark contrast at certain concentrations. At 15% EtOH, unlike at 18h of exposure, cultures treated for 5 min and 2h had higher viability. This difference was most distinct at 20% and continued to 30%. At 40-50% ethanol exposure, the viabilities of all conditions were virtually undiscernible from each other. Taken together, these data demonstrate a clear difference in cellular response to stress. At 20% EtOH, yeast remain >90% viable up to 2h and only exhibit cell death when stress duration is increased to 18h. This is consistent with previous studies on ethanol-mediated cell death in yeast in which similar concentrations of ethanol were used to induce RCD. Also, consistent with the general model of stress vs stress response is the eventual complete loss of viability as the concentration of EtOH stress increased (Figure 21). This threshold (50%) was invariably the same between all conditions and suggests a necrotic threshold.

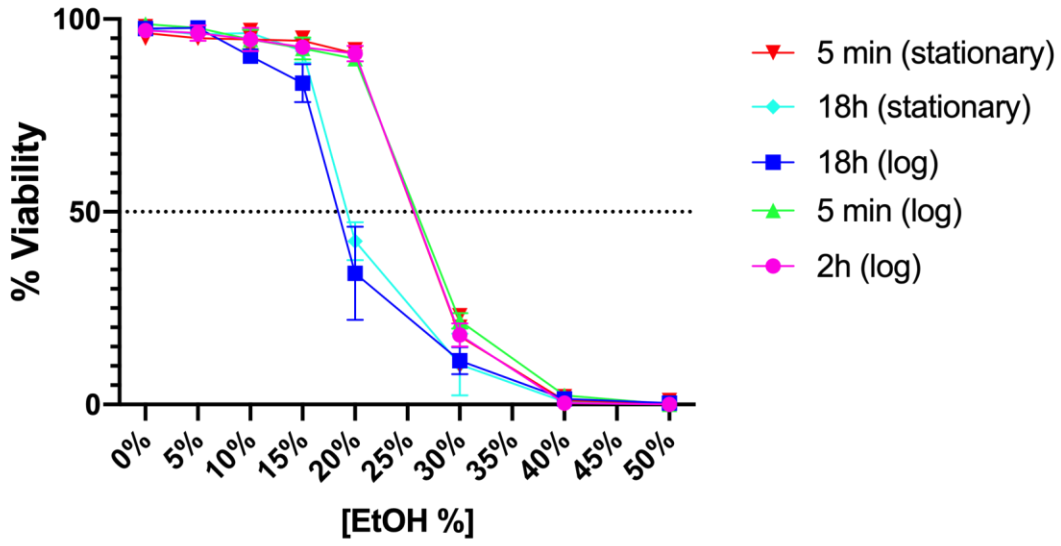

Figure 21. The effect of varying intensities of excess ethanol on yeast viability. *Viability assays were performed with WT yeast using increasing amount of exogenous ethanol. Yeast were treated with ethanol and incubated for 5min, 2h and 18h to additionally assess the effect of stress duration.*

3.3.2 Stress responses to metal salts

Given the response of yeast to increasing ethanol stress and its alignment with traditional stress response models, we asked whether the response to excess metal salts such as copper would be the same. Yeast grown in the presence of increasing amounts of exogenously added copper demonstrated a dose dependent loss of viability (Figure 22). Consistent with previous findings, loss of viability was observed between 1.4mM concentrations of copper up to 2.2mM. Interestingly, while the growth status of yeast had no effect under ethanol stress, stationary yeasts were more resistant to copper stress but nonetheless suffered a dose dependent loss of viability. Surprisingly, up to 5mM copper stress, no additional cell death was observed in either growing or

stationary yeast cultures. To test "extreme" levels of copper stress, 50mM concentrations were used. Interestingly, in neither log phase nor stationary yeast was complete cell death achieved after 18h exposure. Despite a greater than 35x increase in the minimum death inducing concentration of copper, roughly 20-30% of yeast cells remained viable (Figure 22). In accordance with experiments with ethanol, we also measured yeast viability in cultures that only received brief exposures to copper stress to measure potential necrotic events. Surprisingly, unlike under ethanol, yeast maintained near 100% viability at all concentrations of copper stress (Figure 22) for up to 2 hours.

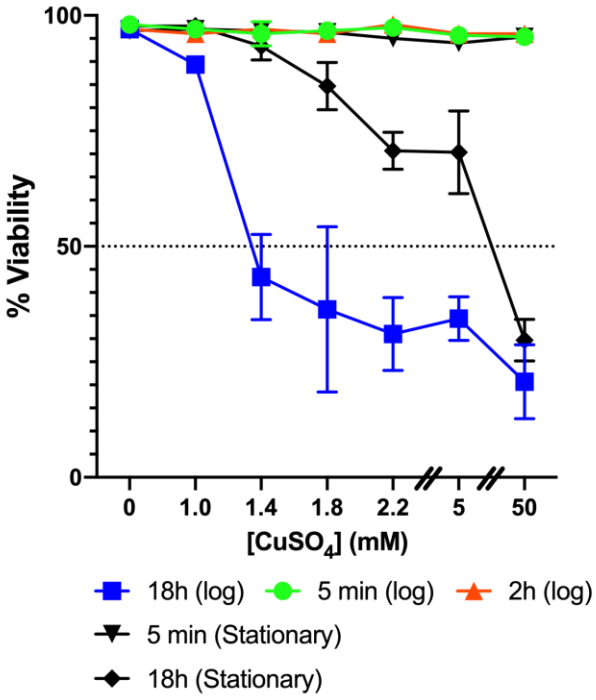

Figure 22. The effect of varying intensities of excess copper on yeast viability. *Viability assays were performed with WT yeast using increasing amount of exogenous ethanol. Yeast were treated with indicated amounts of excess copper and incubated for 5min, 2h and 18h to additionally assess the effect of stress duration.*

These data drastically contrast with what was observed with ethanol stress and contrary to what traditional models of cellular stress vs response predicts. A potential pitfall of these data was that the level of copper might not actually be high enough to induce necrosis. To resolve this, yeast were further treated with 0.5M concentration of exogenous copper; representing a greater than 35000-fold increase in the minimum cell death-inducing concentration. Surprisingly, after 5 minutes of incubation, the viability of yeast cultures did not significantly change. As a control, yeast were incubated at 90C or 50% ethanol for 5 min. Unsurprisingly, cell viability was completely abrogated in either condition.

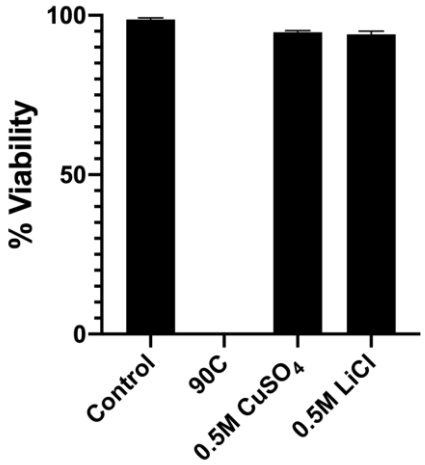

Figure 23. The effect of extreme stress on yeast viability is different between RCD-inducing agents. *The viability of growing cultures of yeast in the presence and absence of stress was measured after 5 minutes of each respective treatment.*

To test whether the difference in yeast response between ethanol and copper is observable with other forms of stress, yeast were also treated with lithium (Fig 23). As previously demonstrated, lithium, like copper is a metal ionic stressor that is capable of inducing RCD in yeast in a dose dependent manner (Figure 18). In the presence of lithium at 60mM, cell death is readily observable after 18h incubation. At 0.5M, like copper, exogenous lithium does not induce cell death within 5 min of exposure (Figure 23). Such similarities with copper and differences with ethanol and temperature stress suggest that extreme doses of certain stresses do not necessarily result in further amounts of cell death nor necrosis and the cellular response cannot necessarily be related to intensity but rather the nature of the stress itself.

Chapter 4.

Discussion & Conclusions

4.1. Summary of principle findings

4.1.1. CSRP3 is a novel LIM-only general pro-survival sequence

In chapter 3.1, it was observed that the heterologous expression of human CSRP3 in yeast could protect against the growth inhibitory and RCD-inducing effects of both ectopic BAX expression as well as excess exogenous copper. In the case of copper, the expression of CSRP3 not only protected against copper-mediated growth inhibition, but also copper-mediated RCD. Furthermore, in yeast, these protective effects did not appear to require autophagy as the deleterious effects of copper could still be protected against in CSRP3-expressing yeast that lack ATG1. In later experiments, it was also shown that CSRP3's range of protection included the growth inhibitory and RCD-inducing effects of excess exogenous lithium.

As a LIM-only protein, it stood to reason that the LIM domain might play a role in mediating CSRP3's pro-survival effect. Analysis of the yeast genome revealed the presence of an additional 4 LIM containing genes – RGA1, RGA2, LRG1 and PXL1. The overexpression of these LIM genes in yeast in the face of copper stress did not confer any discernable pro-survival advantages to growing yeast. Similarly, when knocked out, the absence of yeast LIM genes did not alter the response of yeast to stress except for those lacking PXL1. In yeast lacking PXL1, exposure to sublethal copper and SDS revealed they were more sensitive to stress but could still be protected by both CSRP3 and 14-3-3 expression; suggesting an instance of non-specific synthetic lethality.

In experiments where CSRP3's own LIM domains were mutated, it was observed that the loss of either LIM domain did not affect CSRP3's ability to protect against excess copper. Thus,

the LIM domain itself is unlikely to have pro-survival properties or be necessary for pro-survival responses in CSRP3-mediated stress protection.

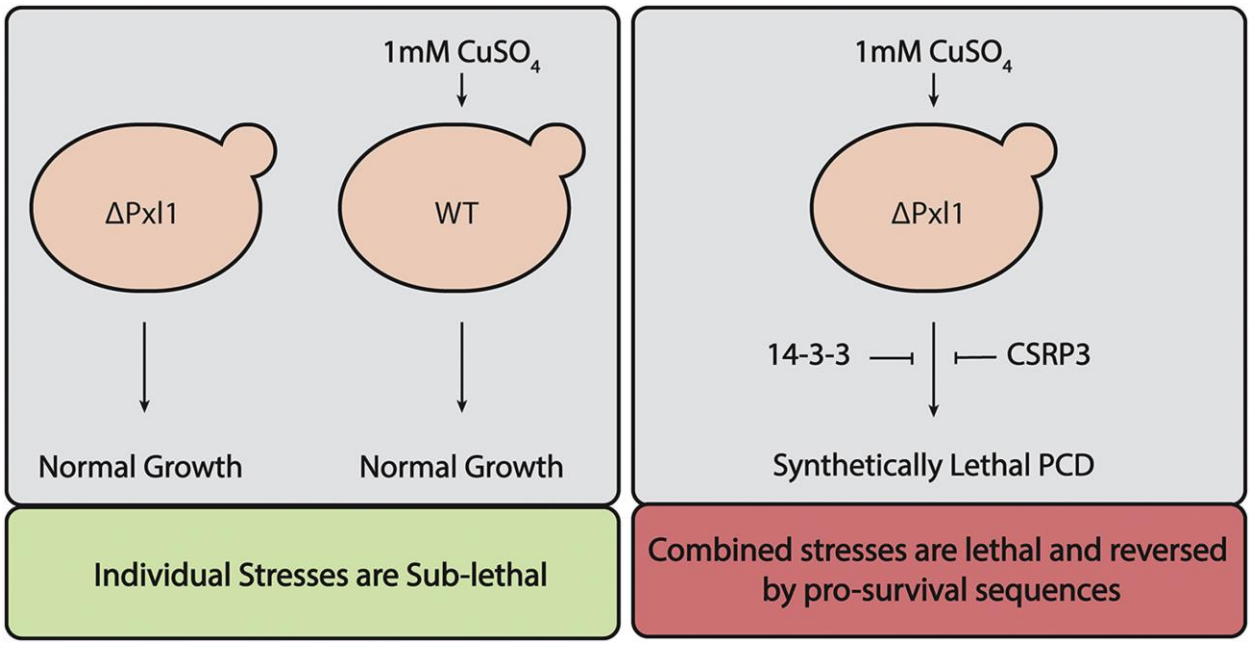

Figure 24. ***Model depicting the relationship between the synthetic lethality of*** **pxl1Δ-*** ***sublethal copper and the induction of Regulated Cell Death (RCD).*** *Cells lacking the* PXL1 *gene or wild type (WT) cells treated with sublethal 1mM* $CuSO_4$ *show normal growth. In contrast, cells lacking the* PXL1 *gene are unable to grow and undergo cell death when treated with sublethal 1mM* $CuSO_4$*. The lethal effects of the combined stresses can be reversed by expressing human CSRP3 or the pro-survival 14-3-3 sequence. This indicates that* pxl1Δ*-sublethal copper mediated synthetic lethality is due to a non-specific effect that serves to induce RCD.*

4.1.2. The heterologous expression of pro-survival sequences in yeast elucidates novel RCS pathways

The use of pro-survival sequences including human 14-3-3 and CSRP3 as tools to investigate novel RCD and RCS responses in yeast was explored in chapter 3.2. Expressing pro-survival sequences in yeast non-specifically protected against some but not all metal stresses. In yeast, it was observed that the response to external iron, copper and lithium stress differed and could be differentiated by the expression of human 14-3-3 and CSRP3. While the expression of these protected against copper and lithium-mediated growth inhibition and cell death, it conversely enhanced the deleterious effects of iron. In experiments with RCD mutants, it was observed that the lack of many key RCD regulators did not affect the ability of 14-3-3 to protect against stress. In the case of VMA3, yeast knocked out for it were found to be sensitive to sub-lethal concentrations of both copper and iron. Interestingly, the expression of 14-3-3 in these yeasts was still able to protect against copper but not iron suggesting that the vacuole is required for iron-mediated RCD. Additionally, when control yeast were preconditioned with sub-lethal levels of copper, their response to lethal levels of iron changed to resemble that of copper, suggesting some degree of crosstalk between iron and copper-mediated stress responses. Lastly, the use of pro-survival sequences was also used to explore stress scenarios involving lithium. Here, CSRP3 was used to examine how previously reported mechanisms of lithium toxicity related to CSRP3's ability to protect against it. In knockout experiments, only in yeast lacking MCK1 was CSRP3 unable to protect against the deleterious effects of excess lithium. These data highlight MCK1 as a target of interest for mechanistic studies of both lithium toxicity and lithium's effect on cells.

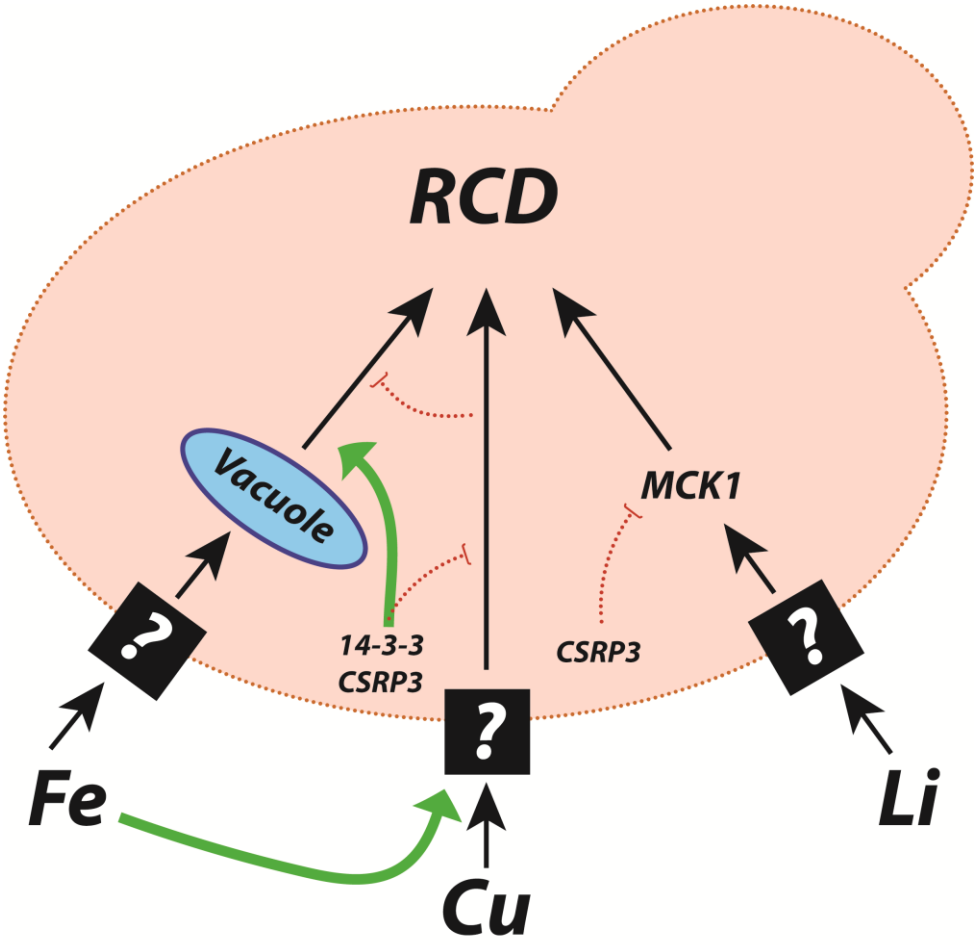

Figure 25. RCD pathways in yeast can be elucidated using pro-survival sequences. *Metal stresses such as iron, copper and lithium induce RCD through distinct subroutines that can be distinguished from one another through the heterologous expression of pro-survival sequences.*

4.1.3. Stress intensity does not uniformly predict stress response

The adage "the dose is the poison" in many cases accurately reflects the relationship between the intensity of stress and the response of cells to it. Indeed, this paradigm has formed the basis of the model of stress intensity vs cellular response and in practice can be readily observed with stresses such as temperature and exogenous ethanol as seen in Chapter 3.3. Yet with other

stresses such as metals salts, while dose dependent reductions in cell viability are both observable and measurable, further increases in intensity do not necessarily lead to a more extreme death response (necrosis) nor complete abrogation of cell viability. Indeed, the complete loss of viability in cultures was unachievable with copper and lithium stress, even at levels thousands of times higher than the minimum required to induce RCD. Taken together, we conclude that for some stresses, intensity cannot uniformly predict stress response.

4.1.4 Iron toxicity involves the precipitation of phosphate in media

In chapter 3.2 the complications of exogenously supplied iron salts in cell culture media was also investigated. Iron is a commonly studied stress and known inducer of cell death in vitro yet its proper study has been in large complicated by its solubility in complex media. In experiments with both yeast and mammalian cell culture media, the formation of a distinct precipitate was observable in a dose dependent manner. The kinetics of this precipitation demonstrated that it formed at concentrations well below the minimum death-inducing concentration of iron. This suggests that the addition of iron as a form of stress into media may be non-specific and instead remove essential media components leading to a kind of deficiency-stress. Based on the stoichiometric availability and known insolubility of iron phosphate as well as previously known literature on the death inducing effects of phosphate depletion, it was concluded that the addition of iron to cell culture media indirectly and non-specifically causes growth restriction and cell death by removing phosphate from media. This notion was further corroborated in experiments that added exogenous phosphate into iron treated media resulting in a dose dependent rescue of growth and viability in yeast.

4.2 Discussion and significance of principle findings

4.1.1. CSRP3 and LIM containing genes

Previous studies have shown that human CSRP3 is a muscle specific gene that plays structural roles involved in contractility, signal transduction and growth regulation. Gene knockout studies in animals have linked the loss of CSRP3 to cardiomyopathy and muscular dystrophy (Cui et al., 2020; Geier et al., 2008; Trump et al., 1980). In gene silencing experiments in myoblasts, the reduction of CSRP3 expression resulted in a diminished autophagic response and a loss of muscle mass in chickens (Cui et al., 2020). Here in yeast, we have observed that the heterologous expression of CSRP3 protects against the growth inhibitory and RCD-inducing effects of both ectopic BAX expression and excess exogenous copper. As far as we are aware, this is the first report on CSRP3's ability to protect against stress mediated RCD and we propose it as a novel pro-survival sequence.

In addition, we are the first to examine the LIM domain in a pro-survival context. LIM domains are evolutionary ancient motifs thought to mediate protein-protein interactions with both LIM and non-LIM containing proteins (Arber & Caroni, 1996; Feuerstein et al., 1994). Here we tested the hypothesis that the LIM domains of human CSRP3 mediates its pro-survival effects by binding to a yeast LIM protein or a non-LIM target. Using genetic approaches, we showed here that despite CSRP3 only having LIM domains, it is unlikely to be specifically involved in mediating pro-survival responses in yeast. This is surprising considering CSRP3's reported role as an adaptor protein playing roles in signal transduction, growth regulation and autophagy (Rashid,

Runci, Russo, et al., 2015). In muscle, CSRP3 serves as a scaffolding protein that organizes interactions at Z-line structures. CSRP3 is also known to directly bind actin as well as prevent cofilin-mediated depolymerisation (Rashid, Runci, Russo, et al., 2015). It would be interesting to see if CSRP3 also performed analogous functions in yeast cells. Following our study, others have examined the loss of CSRP3 in chicken myoblasts and found those cells to be more sensitive to stress-induced apoptosis, suggesting that CSRP3 also plays a pro-survival role in mammalian cells as well (Cui et al., 2020). Nonetheless, it remains obscure as to exactly how CSRP3 specifically or non-specifically mediates a pro-survival response.

4.1.2. Synthetic lethality & fitness

In this thesis, we analysed an instance of synthetic lethality between the loss of PXL1 in yeast and sub-lethal concentrations of excess copper. Synthetically lethal phenotypes often occur when cellular growth or viability is lost due to the combined loss of two non-essential genes in the same cell. This can also arise due to the loss of a single non-essential gene and a sub-lethal level of a stress. In either case, the common explanation for this phenotype is the loss of a common function. In this regard, due to the relatively difficult nature of studying non-essential genes, synthetically lethal interactions have served as popular tools for the elucidation and analysis of gene functions and networks. Indeed, on a genomics level, high throughput analyses of double gene knockouts in yeast has identified all possible combinations of synthetically lethal gene pairs (Costanzo et al., 2016). In some instances, there exists several synthetically lethal combinations for the same gene. In yeast, the loss of cardiolipin synthase (CRD1) is synthetically lethal with over 100 other genes. As a result, cardiolipin is suggested to have highly pleiotropic functions including cell wall

biogenesis, mitochondrial transport and the electron transport chain (Raja et al., 2017; Zhou et al., 2009). While these may point to novel functional redundancies for cardiolipin, alternative explanations exist. *crd1*Δ mutants have elevated levels of ROS suggesting that these cells while viable, are chronically stressed. Thus, it is not surprising that CRD1 is synthetically lethal with several genes that encode antioxidant genes such as superoxide dismutase. These kinds of synthetically lethal combinations are possible instances of decreased fitness rather than the loss of essential gene functions. In other words, a potential limitation of synthetically lethal gene pairs is the assumption that a loss of growth or viability is the result of a combined defect in a specific cellular process rather than an overall decreased in fitness.

The observations made in this thesis suggest that the interaction between the loss of pxl1 and sublethal copper is unlikely to underlie a specific process between the two. In fact, the lethal effects of excess copper is rescuable by the expression of both CSRP3 and 14-3-3. Furthermore, given that CSRP3 and 14-3-3 protect against many other stresses, the combination of sub-lethal copper and loss of PXL1 suggests a case of RCD activation. This is consistent with our previous observation that the sensitization to copper and iron stress in yeast lacking VMA3 is also due to the non-specific induction of RCD (Eid, Zhou, et al., 2017). Indeed, if copper were truly specifically causing RCD, no amount of pro-survival should block it. Instead, it is likely that pxl1Δ like vma3Δ and crd1Δ have a decrease in overall fitness. While it is unclear as to how the expression of a pro-survival sequence can protect against this, a possible explanation is that the expression under GAL1 may serve as a pre-conditioning mild stress that stimulates cytotoxic processes that can promote growth and viability at these concentrations.

In this regard, some instances of synthetic lethality can be explained by examining overall fitness. It can be envisioned that many non-essential genes are functionally important but their loss

can be compensated for by redundant or regulatory mechanisms allowing cells to grow normally under laboratory conditions. It is plausible that these mutants are in fact in a state of chronic mild stress. This would explain why many single gene knockout mutants are relatively more sensitive than control to agents that induce oxidative stress (Tucker & Fields, 2004). Furthermore, sublethal stresses are commonly found to synergize with other sub-lethal stresses and lead to cell death. Taken together, synthetically lethal combinations may or may not be specific and the heterologous expression of pro-survival sequences such as human CSRP3 or 14-3-3 could be used as tools to differentiate between the two.

4.1.3. Implications for cellular stress-cellular response relationships

The most basic interpretation of the relationship between stress and the response of cells to it is a graded one where the intensity of stress dictates the response. Despite its success at explaining the cellular response to some stress like ethanol and temperature, it does not explain why thousands-fold concentrations of excess copper or lithium cannot rapidly abrogate cellular viability. The difference in response to stress may relate more to the nature of the stress itself.

Ethanol is a widely used cleaning agent owing to its microbicidal and viricidal properties (Centers for Disease Control and Prevention, 2008). It can both freely and quickly equilibrate between the cell and the extracellular space as it is cell permeable (Ingram, 1990). In bacteria, ethanol kills in two ways: protein denaturation and dissolving the lipid membrane (Centers for Disease Control and Prevention, 2008). In mammalian stem cells, the metabolism of ethanol is reported to also involve the production of genotoxic metabolites, increases in ROS, induction of DNA damage, interference with DNA repair and protein denaturation (Di Rocco et al., 2019). In

yeast, the use of ethanol stress has been shown to induce cell death accompanied by morphological changes like that of apoptosis (Kitagaki et al., 2007). Similarly, high temperatures irreversibly denature and breakdown structures and molecules alike. Like ethanol, extreme levels of heat damages and destroys biomolecules in an uregulated manner leading to an unregulated type of cell death - necrosis. Thus, it is likely that true necrotic stresses involve immediate irreversible damage beyond cellular control. For others like copper, it is plausible that cells actively resist equilibrating with excess stress, but they can however choose to die in response.

In our experiments with metal stresses, we see that the resulting cell death does not necessarily relate to the intensity of stress. For example, with copper stress, a dose of 5mM to 50mM does not produce a significantly different outcome (Figure 22). In fact, increasing the level of stress to 0.5M for any of the metal stresses we tested does not induce cell death within 2h. In fact, in either stress scenario, cells are invariably still alive, able to persist in these high stress environments.

Fundamentally, these data suggest two things. Firstly, homeostasis cannot be overcome simply by increasing concentrations. This is evident in experiments where in the presence of stock concentrations of copper, no change in cellular viability was observed up to 2h. In fact, even after 18h exposure, some cells were still alive suggesting that they continue to resist their toxic environment. Secondly, the nature of the stress itself may be more related to the stress outcome rather than its intensity. Both temperature and ethanol can freely equilibrate between intra and extracellular compartments and cause damage. While excess copper is related to an increase in oxidative stress inside cells, the mechanism by which copper is transduced to ROS is unclear (Zhou, Eid, Boucher, et al., 2019; Zhou, Eid, Miller, et al., 2019). It is of interest that a recent high impact study of copper toxicity deliberately employed ionophores to raise intracellular

concentrations to study its effects (Tsvetkov et al., 2022). This echoes key principles of cellular homeostasis and shows that it cannot be overcome simply by increasing concentration. We have argued in recent reviews that stress is an agonist for the induction of RCD and likely involves signal transduction mechanism beginning at the membrane rather than entering the cell (Zhou, Eid, Boucher, et al., 2019; Zhou, Eid, Miller, et al., 2019).

4.1.4. Implications for iron toxicity

Iron is an interesting essential trace element as its ability to accept and donate electrons is thought to facilitate its own toxicity (Eid, Arab, et al., 2017; Winterbourn, 1995). Here we showed that yeast can grow in the presence of excess iron as high as 5mM; representing a ~4000-fold excess of iron relative to what exists in minimal YNB media (Figure 12). This suggests that large excesses of iron are not necessarily cytotoxic. Only at concentrations beyond 7mM or a ~6000-fold relative increase in iron are growth and viability affected (Figure 12). Taken together, these data call into question whether excess iron is actually toxic.

Doubts about specific iron toxicity have been investigated in previous studies that measured intracellular levels of iron in cells grown with excess extracellular iron. Holmes-Hapmton et al. found using biophysical techniques that the change in intracellular iron levels was not significantly changed despite a 250-fold increase in extracellular iron (Holmes-Hampton et al., 2013). As far as we are aware, despite many studies, the increase in intracellular iron in response to excess extracellular iron has never actually been observed. Instead, in response to external stress, intracellular stores of iron may be released to serve as stress-responsive secondary messengers (Zhou, Eid, Boucher, et al., 2019; Zhou, Eid, Miller, et al., 2019).

While we use mM concentrations of iron to induce cell death, others have used uM ranges that are normally non-lethal. Interestingly, our review of iron toxicity literature has found that many studies additionally involve another sub-lethal stress to generate "iron toxicity" effects (Eid, Arab, et al., 2017; Lin et al., 2011). Additionally, many iron toxicity studies rarely use iron at the concentrations studied in this thesis. The reason for this is likely due to its low solubility in complex cell culture medias. Serum starvation or deprivation, a known apoptosis inducing stress is most commonly combined with sub-lethal iron in mammalian cultured cells (Aoki et al., 2004; Reza Ardehali et al., 2011; Portt et al., 2011). Although the combined stresses are both required to induce cell death, it is more likely to be synthetic lethality than specific iron mediated cell death.

In mammalian cell studies, the use of serum starvation is commonly paired with added iron albeit at significantly lower and probably non-precipitous concentrations of iron than those used in this study. Yeast models of iron toxicity have also been widely studied but frequently involve the use of iron-sensitive mutants to induce cell death (Zhou et al., 2020). In yeast, iron often studied in mutant cells that are characterized as iron supersensitive (Lin et al., 2011). In our hands, the iron sensitivity of some of these mutants are not restricted to iron but can also be observed with other stresses like copper (Eid, Zhou, et al., 2017). For example, yeast lacking VMA3 show sensitivity to both excess copper and iron (Figure 10). In addition, the cell death observed here can be non-specifically protected against by a number of pro-survival sequences. Taken together, it might be that are not examining iron mediated cell death, but instead, cell deaths that are more akin to synthetic lethality.

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