Investigating the Role of TOR Signalling, and the Link between Actin Binding Protein Cofilin and Mitochondrial Function and their Effects on Recombinant Protein Production

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Declaration

No part of this thesis has been submitted in support of any other application for a degree or qualification of the University of Kent or any other University or Institution of learning.

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Acknowledgments

Contents

Abbreviations

aa	Amino acid
ABC	ATP-binding cassette
APS	Ammonium persulfate
ATG	autophagy specific gene
ATP	adenosine triphosphate
Bp	base pairs
CHO	Chinese Hamster Ovary
Da	Daltons
DEPTOR	DEP domain-containing mTOR interacting protein
DNA	deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Enhanced chemoluminescence
EDTA	Ethylenediaminetetraacetic acid
EGO	Exit from rapamycin-induced growth arrest
ER	Endoplasmic reticulum
ERAD	ER associated protein degradation
et al	ET allia (and others)
ETC	electron transport chain
IMS	intermitochondrial space of membrane
MAPK	Mitogen Activated Protein Kinase
mRNA	messenger ribonucleic acid
mt DNA	mitochondrial DNA
MW	Molecular weight
NADH	nicotinamide adenine dinucleotide
OD	optical density

OXPHOS	oxidative phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PAS	Pre-autophagasome structure
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PEG	Polyethylene glycol
PGK	Phosphoglycerate kinase
PKA	Protein kinase A
PMSF	Phenylmethanesulfonyfluoride
Pptox	preprotoxin
PRAS40	prolin-rich Akt substrate 40
PTM	Post translational modification
PVDF	Polyvinylidene fluoride
qRT	Quantitative real-time
Raptor	Regulatory-associated protein of mTOR
Rictor	Rapamycin-insensitive companion of mTOR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rP	Recombinant protein
rpm	rotations per minute
RT	Room temperature
S6K	Ribosomal protein S6 kinase
SC	synthetic complete (minimal medium)
S.cerevisiae	*Saccharomyces cerevisiae*
S.pombe	*Schizosaccharomyces pombe*
SDS	sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA

siRNA	small-interfering RNA
SRP	Signal recognition particle
TAE	Tris Acetate EDTA
TEMED	Tetramethylethylenediamine
Tris	2-Amino-2-2hydroxymethyl-propane-1,3-diol
mTOR	Mammalian Target of rapamycin
TOR	Target of rapamycin
TSC1/2	Tuberous sclerosis complex
tRNA	Transfer ribonucleic acid
UPR	Unfolded protein response
YPD	yeast extract peptone dextrose
YPG	yeast extract peptone glycerol
YT	yeast extract tryptone

Abstract

Due to the increasing demand in Biotherapeutics, this thesis focussed on investigating novel ways to exploit major signalling pathways to enhance recombinant protein production (rP) in yeast and CHO cell expression systems. The expression of model recombinant proteins in yeast allowed us to screen for specific targets located within important environmental signalling pathways for effects upon recombinant protein production. Targets identified within the yeast system and that were conserved were then tested within CHO cell expression system.

One of the systems investigated with regards to rP production was target of rapamycin (TOR) pathway. TOR signalling is a global regulator of nutrient sensing and energy status. It is a highly conserved Ser/Thr protein kinase pathway which is involved in processes such as protein synthesis, ribosome biogenesis, cell cycle, gene transcription, autophagy, and metabolism. As TOR is heavily involved in the control of protein synthesis, we asked whether it could be manipulated to enhance recombinant protein production (rP). We identified that TOR mediated amino acid sensing and autophagy are important for maintaining rP production in both yeast and CHO cells systems.

Previous work supporting this thesis suggests that the actin binding protein cofilin can be manipulated to enhance both mitochondrial function and environmental sensing. We hypothesised that Cofilin functions may be useful in maintaining mitochondrial function during the stress imposed by rP production. Using a library of Cofilin strains expressing mutant alleles, we found that the control of MAPK signalling, and in particular the downstream effector Ste12, and mitochondrial functionality have a significant effect on GLuc expression when driven by the mating factor signal sequence. Manipulation of mitochondrial function or STE12 function also led to changes in rP production. In many case changes in Gluc expression or secretion were not replicated in a separate Killer toxin secretion system. As we did not see the same effects in alterations to killer toxin secretion, we concluded that these findings were specific to the presence of the signal sequence from the mating alpha factor. The manipulation of MAPK signalling and mating factor signal sequence driven secretion would appear to be a promising avenue for further enhancement of the yeast system.

This work highlights the complex nature of manipulating signalling networks to achieve improvements in rP yield. However a better understanding of the mechanisms involved and the ability to manipulate these changes/responses will be beneficial for enhanced protein production from yeast and mammalian expression systems.

Key words: cofilin, MAPK, Target of Rapamycin (TOR), recombinant protein production, autophagy, amino acid sensing, *Gaussia* luciferase (GLuc), killer toxin, mating alpha factor.

Chapter 1

Introduction

1.1 Introduction to recombinant protein production

1.1.1 The rise of recombinant biopharmaceuticals

Recombinant proteins are produced from various expression systems by recombinant DNA (rDNA) technology, with the demand for heterologous gene products produced in the biopharmaceutical industry rising due to an increasing need for healthcare. The biopharmaceutical sector encompasses a range of products that can be used for therapeutic applications. In 1982 the first biopharmaceutical Humulin, a recombinant human insulin, was produced in *Escherichia coli* and approved for use to treat patients with Type I Diabetes (Walsh, 2010a).

Now, recombinant protein production is a multibillion-dollar market producing proteins such as interferons, erythropoietin, vaccines, industrial enzymes and antibodies (see figure 1.1). Although there is an increasing market for biopharmaceutical proteins, there is also a large market for industrial enzymes such as proteases, carbohydrates and lipases for animal feed, biofuels and pharmaceutical enzymes (Mattanovich *et al.*, 2012). Recombinant proteins are also heavily used in research studying proteins involved in disease and to further our understanding in the molecular and cell biology of various organisms. For example, enzymes are needed for digesting DNA in cloning and the use of antibodies in western immunoblotting and enzyme-linked immunosorbant assays (ELISA) which are some of the most commonly used laboratory procedures.

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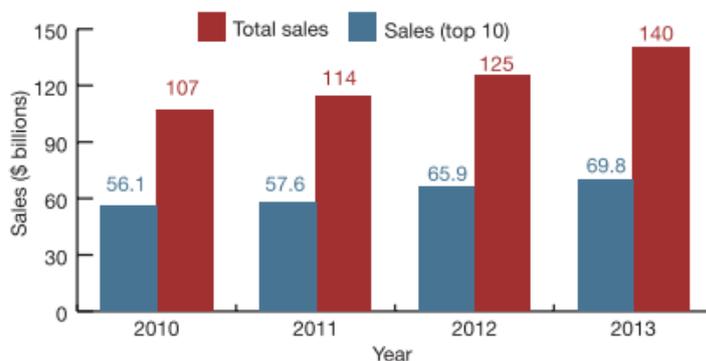
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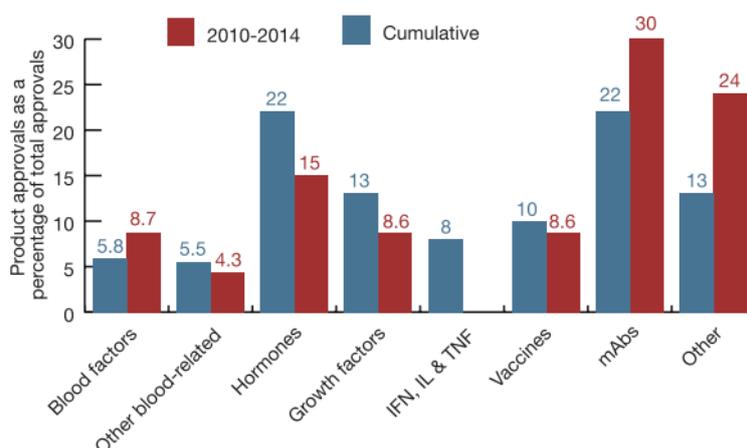
Figure 1.1 Annual biopharmaceutical sales and product approvals. A) Annual sales (total sale values and sales from top 10 selling products between 2010 and 2013). B) Classes of products approved between 2010-2014 and cumulative (between 1982-2014) as percentage of total approvals. Data from the EU and US markets (Walsh, 2014).

When comparing various products recently approved, monoclonal antibody (mAbs) production has increased to the largest class of protein produced, growing from 11% of total approved biopharmaceutical products in the 80's to 26.5%. Outside of these insulin is one of the most profitable non-antibody-based products generating $21.5 billion in 2013 (Walsh, 2014). Antibody-based drugs are a fast growing class of protein therapeutics as the development of expression and secretion of functional non-glycosylated antibody fragments from *E.coli* through antibody engineering have been developed with high titre production. Although the majority of antibodies are 'native' unmodified IgG molecules, there have also been some approved with radionuclides or cytotoxic drugs (antibody-drug

conjugates) attached for the treatment of cancer (Carter, 2011). For highly selective antigen binding, this is dependent on the interaction of the Fc region (base of the antibody) for antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) (Chan and Carter, 2010).

Initially a recombinant protein expression system needs to be investigated to understand their physiology so that this may be exploited, then sourcing appropriate genetic elements for incorporation/engineering into these systems to meet the needs of industry. A number of criteria need to be considered in undertaking engineering strategies such as protein characteristics, quantity produced, time required, purity of the protein, cost effectiveness, compliance with safety requirements and intellectual property (Sodoyer, 2004). However, as well as considering improvement of recombinant protein expression by genetic modification, many parameters that may affect the health of the cell, for example giving the correct nutrients, oxygen levels and temperature, should be considered. Media optimisation has been shown to dramatically improve titres of recombinant proteins as well as protein quality of culture media, boosting the cell's growth and productivity (Gawlitzek *et al.*, 2009; Jordan *et al.*, 2013). This is not a simple task in mammalian cell culture, as media that is defined and serum free can have 50-100 components with very precise control of parameters such as pH and osmolarity, eliminating the need for concentrated stock solutions which cause instability issues (Jordan *et al.*, 2013).

Although the knowledge in the field of recombinant protein production and the host cell systems utilised is consistently evolving, there is no perfect expression system that can be always used; every protein has individual characteristics which determine which expression system will yield optimal expression and downstream stability characteristics (Sodoyer, 2004).

1.1.2 Advantages to varied expression systems and their genetic manipulation for recombinant protein production

The production of rP has been examined in a variety of prokaryotic and eukaryotic systems yet only particular hosts are exploited within industry. *E.coli*, yeast and mammalian cells are the most widely used along with insect cell lines and transgenic animals (Walsh, 2010a). Approximately 60-70% of rP production is currently performed in mammalian cell lines, including Chinese hamster ovary (CHO) cells, mouse myeloma (NS0), baby hamster kidney (BHK), human embryo kidney (HEK-293) and human retinal cells.

The development of all expression systems is a very dynamic field with new research addressing specific limitations found amongst different systems. New tools to allow genetic modification and re-engineering of existing biological pathways are being investigated. Genetic manipulation can include the ablation, repression or increased expression of specific genes that may in turn alter a cell's ability to produce rP. For example, improvements have generally relied on random mutagenesis or classical breeding techniques to produce cell lines capable of supporting elevated rP production. However, techniques to precisely modify promoter strength of a desired gene, knock out or knock-down/up gene expression and make precise changes to gene sequences are now all techniques readily applied within research labs and industrial labs around the world.

1.1.2.1 Optimising the expression vector

There is much to consider when there are so many variables that limit productivity, both due to the host selected or the product being produced (Hohenblum *et al.*, 2004). Enhancements in expression in mammalian systems for example have been made through good vector design by using alternative signal peptides, strong promoters, alternative/enhanced gene codon selection, specifically selected introns and the use of transcriptional enhancement regions (for example cytomegalovirus, CMV) (Zhu, 2012).

To improve expression levels, strong, tightly regulated promoters are used which are homologous to the species as heterologous promoters can sometimes yield bad efficiency of expression (Mattanovich *et al.*, 2012). For example in yeast, the constitutive GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or Gal1-10 which is induced by galactose whereas in *E.coli*, heat-shock promoter *hsp60* is used and a common inducer is the sugar molecule isopropyl-beta-D-thiogalactopyranoside (IPTG) (Mattanovich *et al.*, 2012). Auto-inducible promoters which are upregulated with changes to culture media can occur near the end of log growth phase. The advantage being that this process doesn't require intervention or the addition of inducers (Sodoyer, 2004). Other factors to consider as potential bottlenecks are codon usage of the recombinant gene, translations signals, translocation of the protein determined by the secretion signal peptide, processing and folding in the ER and Golgi before it is secreted out of the cell (Hohenblum *et al.*, 2004).

1.1.2.2 The use of mammalian model systems

Mammalian cells are the most widely used model system for producing human proteins due to the increasing need for specific post-translational modifications, in particular glycosylation (discussed in more detail below) and are estimated to produce approximately 36% of approved recombinant proteins (see figure 1.2). When comparing quantitatively, microbial production however still produces

more recombinant protein, estimated at 17.9 metric tonnes (68%) in 2010 with 8.5 metric tonnes (32%) derived from mammalian systems (Walsh, 2014).
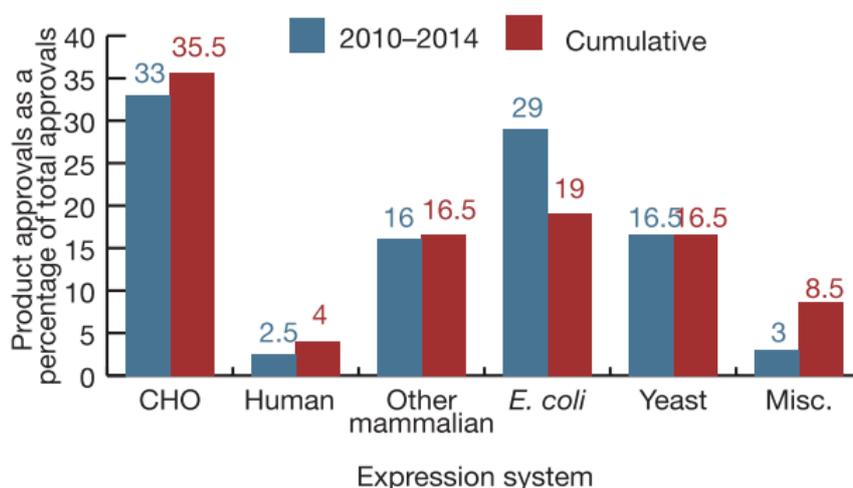
Figure 1.2 Expression systems used in the manufacture of biopharmaceutical products. Cumulative products approved as a percentage of the total between 1982-2014; and between 2010-2014 for more recent approvals (Walsh, 2014).

The mammalian cell lines employed for rP production can be either adherent or cultured in suspension, with suspension cells being more common due to their capacity for single-cell suspension growth (Wurm, 2004). CHO systems in particular have robust proliferation in large-scale suspension culture, are easily adaptable to serum and serum free media, have high cloning efficiency and are easy to manipulate (Fan *et al.*, 2012). Industry benefits from mammalian cell expression systems as many of the rP products that are designed for therapeutic use require complex protein processing and post-translational modification that cannot be accomplished by bacterial or native yeast systems. However, drawbacks include high costs due to the requirement for complex media (Hamilton and Gerngross, 2007).

After synthesis of therapeutic proteins, the majority require post-translational modification (PTM) for therapeutic application which can vary between organisms, the most common being glycosylation, carboxylation, hydroxylation, amidation and sulfation, proteolytic processing and disulphide bond formation. Eukaryotes can undertake covalent modifications and many have been characterised to influence structure and function of the protein and so are essential to its activity (Walsh, 2010a). For example *N*-glycosylation is important for protein folding*, in vivo* half-life and function, and is often

needed in the production and secretion of glycoproteins. Overexpression of glycosyltransfereases for example in CHO cells leads to the enhancement of glycan quality (Andersen and Krummen, 2002; Zhu, 2012). Although glycosylation is possible in yeast and fungi for example, it can often be different in microorganisms producing heterogenous high mannose type N-glycans (which are immunogenic to mammal cells) compared to mammalian cells which have N-glycans which are mainly complex or hybrid type (De Pourcq *et al.*, 2010).

Most mammalian cell lines will produce glycoproteins containing *N*-glycolylneuraminic acid (Neu5Gc), which in humans is absent, leading to an immune response if subjected to the glycoprotein. Although CHO cell lines contain lower levels of Neu5Gc sialylation compared to murine cell lines, Neu5Gc still needs to be controlled (Xu *et al.*, 2011). It was discovered that the level of Neu5Gc can be controlled through environmental factors such as the addition of sodium butyrate (SB) that decreased the levels of Neu5Gc by 50-62%. The same study also reported that Neu5Gc levels were lowered by 59% with a low temperature shift closer to stationary phase which is commonly used to increase culture duration and titre in industry. This likely changes metabolic states effecting NADH levels, and cells accumulate at different phases of the cell cycle (Borys *et al.*, 2010). Sodium butyrate (SB) is a short chain fatty acid shown to modify oligosaccharide content of glycoproteins, it was suggested that SB can cause changes to sialylation with the ability to effect the recycling of sialic acid from existing glycoproteins, bypassing *de novo* synthesis (Kreuter, 1996; Borys *et al.*, 2010).

A number of conventional gene knockouts by homologous recombination in CHO cells have been useful in creating high rP producers. An important tool used for silencing gene expression in cells or organisms is RNA interference (RNAi). This technology is used to increase cellular productivity and improving the quantity of rP production. RNAi is induced by small double-stranded RNA molecules which can negatively regulate gene expression. Genes can be silenced using siRNA (small interfering RNA) which are a class of double stranded RNA molecules or by the use or shRNA (short hairpin RNA molecules) which are encoded by plasmids or viral vectors. These are structurally related to microRNAs which are produced by the cell to regulate gene expression (S.C. Wu, 2009). However, for knockdown in mammalian cells, shRNA-expressing DNA is delivered to the nucleus to be transcribed whereby it is then processed by Dicer-dependent cleavage in the cytoplasm into siRNA. This siRNA is incorporated into the RNAi-induced silencing complex (RISC) which released the complementary strand to the target mRNA and is degraded. With the target mRNA, RISC finds the complementary sequence and cleaves the mRNA (Hannon and Conklin, 2004; Mikuma *et al.*, 2004; Takahashi *et al.*, 2009).

However, latest genome editing includes the use of double-stand break (DSB)-inducing nucleases in targeting specific sites, such as zinc finger nucleases (ZFNs) or clustered regularly interspace, short palindromic repeat (CRISPR) technology from bacteria. CRISPR generates DSBs using the RNA guided Cas9 endonuclease, using base-paring between the target DNA and the engineered RNA (Mali *et al.*, 2013; Sander and Joung, 2014). An advantage to this technology is the ability to simultaneously introduce multiple gene mutations as multiple guided RNA can be used. These DSB introduced by endonucleases are repaired by the cell through two different pathways; the first is known as nonhomologous end joining (NHEJ) where random mutations occur disrupting the gene, the second is homology-directed repair (HDR), where precise point mutations are introduced using a donor template(Sander and Joung, 2014).

Engineered cell lines can be created transiently (introducing a plasmid DNA) or have the cells secrete the rP permanently in a stable cell line which need to be isolated and characterised. Although transient expression is the less time consuming option, scalability of this process is limited due to culture surface needed and the fact the plasmid is lost from the cell during division result in low yields (Durocher *et al.*, 2002; Pham *et al.*, 2006)(Agrawal & Bal, 2012). There have been attempts to scale–up transient cell line manufacturing by growing them in roller bottles, as well as transferring these adherent cells to microcarriers or suspension culture, although these methods had limited success (Pham *et al.*, 2006). Transient production has been scaled up to more than 150 litre volumes, generating grams of antibodies, suitable for small scale production for antibody screening and therefore does now offer an option for the rapid generation of early stage material (Baldi *et al.*, 2007; Jäger *et al.*, 2013).

For CHO cells in particular, the development of high producing cell lines is slow due to the cell line generation and selection processes where thousands of individual cell lines are screened to obtain a sufficient number which exhibit desirable productivity and cell growth. For commercial biotherapeutics, stable clone selection with high productivity takes several months at least. Once a reliable host has been selected, the expression system needs optimisation, with media development and nutrient feeding strategies before the high producers are selected. This classic selection process method is where roughly one cell is deposited in each well of a microplate with conditioned media for good growth conditions; however this limits the number of clones screened and is inefficient (Agrawal & Bal, 2012). More recent methods for selection include the use of fluorescence-activated cell sorting, and measurement of the relative mRNA transcript levels. It is preferred that the selection process is performed early in clinical development of the product as this avoids any potential changes in the glycosylation profile which is important in antibody production for example (Shukla and Thömmes, 2010).

1.1.2.3 The use of yeast as model systems

Microorganisms offer advantages over mammalian cell systems in that they can be grown and evolved rapidly, are much cheaper to grow in culture and are relatively easy to modify by genetic manipulation. Thanks to advances in genetic engineering, both prokaryotic and eukaryotic microorganisms such as the yeast *S.cerevisiae* and *E.coli* have been used successfully to produce heterologous proteins for industrial processes. However, as with mammalian systems, micro-organisms also possess drawbacks (discussed below).

In yeast, the two major expression systems used are *Saccharomyces cerevisiae* and *Pichia pastoris* (Andersen and Krummen, 2002). Yeast cells grow to high cell densities, are easily maintained in suspension culture and being eukaryotic, are capable of performing glycosylation reactions. Although as mentioned above, they produce naturally high mannose type N-glycans, re-engineering of the N-glycosylation pathway in yeast creating 'humanised' glycoproteins is possible (De Pourcq *et al.*, 2010). All model systems in rP production have their limitations, in yeast, folding and disulphide bond formation are an issue as the chaperone BiP (binding protein) is induced which is a signal for the unfolded protein response (UPR) (described in more detail later on) (Hohenblum *et al.*, 2004; Bravo *et al.*, 2012). Therefore, the secretion capacity of yeast systems may also be less than for mammalian systems (Idiris *et al.*, 2010).

Specific yeasts, such as *Zygosaccharomyces bailii* are capable of growing in highly acidic conditions (pH 2), while *S.cerevisiae* also have a high tolerance to environmental stress such as low oxygen levels giving these microorganisms an advantage where multiple stresses can affect an organism's ability for optimal rP production (Nielsen and Arneborg, 2007; Çelik and Çalık, 2012).

S.cerevisiae have been used and developed in the use of baking, brewing and wine and are one of the best characterised systems and regarded as a safe organism to use. This organism is also believed to have one of the most comprehensive experimental data sets available on the internet such as the *Saccharomyces* Genome Database (SGD) (Çelik and Çalık, 2012). In industrial fermentation, *S.cerevisiae* can grow in low pH and in high sugar and ethanol concentrations with the ability to grow anaerobically with resistance to high osmotic pressure and oxidative stress. This yeast has the potential with genetic engineering for the massive production of biofuels such as bioethanol but also non-fuel products such as glycerol, pyruvate, and organic acids (Mattanovich *et al.*, 2012). In terms of rP production, *S.cerevisiae* has been used to produce human insulin, hepatitis vaccines and human papillomavirus (HPV) vaccines. This yeast has been used as a model organism in investigating the cell and molecular biology behind protein secretion and in strategies for engineering in improving protein

production. This involves selecting the best signal sequence, using the best expression vector and engineering the organism for better folding and PTMs (Idiris *et al.*, 2010; Hou *et al.*, 2012).

Pichia pastoris is another species of yeast commonly used in recombinant protein production, such as secreting high levels of human serum albumin. This species of yeast is methylotophic and so can use methanol as a carbon source when carbon is unavailable. The gene alcohol oxidase 1(AOX1) for methanol utilisation has one of the strongest and tightly controlled promoters in eukaryotes which has been used for expressing a variety of proteins (Hartner *et al.*, 2008; Çelik and Çalık, 2012). During fermentation, *P. pastoris* prefer respiratory instead of the fermentative mode of growth and so there is no build-up of fermentation products such as ethanol and acetic acid enabling culture to grow to a higher cell density making them a good candidate for rP production. Compared to *S.cerevisiae, P.pastoris* secrete proteins with high molecular weight, with low levels of endogenous proteins secreted ensuring simpler purification (Çelik and Çalık, 2012). *P.pastoris* also have the ability to glycosylate (*S.cerevisiae* have hyperglycosylation) with correct disulphide bond formation and proteolytic processing. Additionally, this yeast been reported to produce humanised glycoproteins and engineered to produce a more effective recombinant rat erythropoietin secreted, raising hematocrit levels compared to the wild type *P.pastoris* (Hamilton *et al.*, 2006; Çelik *et al.*, 2007). This strain was able to secrete human glycoproteins with fully complex terminally sialylated N-glycans by removing genes for yeast-specific glycosylation and introduced heterologous genes to replicate human glycosylation (Hamilton *et al.*, 2006).

1.1.2.3 The use of E.coli as a model system

Escherichia coli (E.coli) is the most common choice for production of recombinant proteins in microorganisms, being used for both studies to gather information on microbial physiology and the use of molecular tools such as engineered phages, gene expression cassettes and plasmids (Ferrer-Miralles *et al.*, 2009). Many heterologous proteins have been produced within the periplasm that possesses an oxidising environment where disulphide bond formation occurs which are necessary in proteins such as antibody fragments, many peptide hormones and enables proper folding of proteins into their native conformation (Schlegel *et al.*, 2013).

A major drawback of using *E.coli* systems has been in the production of extracellular protein which reduces downstream processing needed, however lab strains and engineered strains have the ability to secrete proteins via dedicated secretion systems that naturally exist and co-expression of a lysis-promoting protein (Ni and Chen, 2009; Chen, 2012). In the past saturating Sec-translocon capacity (the protein-conducting channel for heterologous protein to reach the periplasm) has been a major

bottleneck, which mediates the transfer of secretory proteins across the cytoplasmic membrane. Therefore adjustment in the expression levels of the gene of interest so that the Sec-translocon capacity is no longer saturated, reducing toxin effects has been undertaken (Schlegel *et al.*, 2013). Remarkably, due to engineering and high throughput screening, the level of secretion had reached several grams per litre by 2009 with the highest cases being proteins directed to the periplasm, however the exact mechanism by which proteins cross the outer membrane is unknown (Ni and Chen, 2009).

Due to differences in the chemical environment in bacteria and the ER in eukaryotes, even if recombinant proteins are made they may not be correctly folded (Overton, 2014). *E.coli* expression systems often yield insoluble products that are deposited within inclusion bodies which then require downstream processing to recover folded and active product (Porro *et al.*, 2005). Until recently it was thought that glycosylation of recombinant proteins in *E.coli* was not possible due to their cellular structure lacking the ER and Golgi apparatus (sites for post-translational modification). Recently, the N-linked glycosylation system in Gram-negative bacterium *Camplobacter jejuni* was discovered and subsequently transferred into *E.coli* (Wacker *et al.*, 2002; Chen, 2012)*.* However, at present the system yields glycosylation that is often incomplete and most recombinant proteins are not glycosylated at all (Chen, 2012).

1.1.2.4 The use of insect cells and plant based systems

Insect cell lines such as SF-9 and High-Five can produce protein with some theoretical advantages over other expression systems such as viral safety. Another interesting adaptation is that they have the genetic potential to perform sialyation of glycoproteins but this requires metabolic engineering (Sodoyer, 2004). Overexpression of galactosyltransferases and sialyltransferases led to success in generating sialylated oligosaccharides on proteins derived from insects (Andersen and Krummen, 2002). Cultured insect cells are used as hosts for recombinant baculovirus infection, however the production of viral vector for gene expression is time-consuming, cell growth is slow and growth medium expensive (Ferrer-Miralles *et al.*, 2009). Although genetic engineering has been used to select transgenic cell lines, there have only been three products approved, an influenza vaccine Flubok, Provenge treating prostate cancer, and Cervarix for the human papilloma virus (HPV) vaccine (Walsh, 2014).

Plant-based systems have much potential due to the expected high yields, being cost effective with media which is simple, cheap and well defined with a strong viral safety component. Plants have the potential to become an 'edible vaccine' but come with much controversy (due to issues with proof of

principle as well as standardisation of antigen dose) (Sodoyer, 2004). Improved plant production is due to improved production practices and varieties of plant generated through plant breeding, tissue culture and biotechnology. Nevertheless there are additional challenges due to concerns over the release of genetically engineered plants into the environment (Mattanovich *et al.*, 2012).

The first recombinant protein produced was human growth hormone from a tobacco plant, however since then antibodies, the surface antigen of Hepatitis-B-virus, industrial enzymes and milk proteins have been produced in plants (Ferrer-Miralles *et al.*, 2009). Plants also have the capability for post-translational modifications, however proteins produced in plants can be hyperglycosylated, containing sugar moieties immunogenic to humans (Walsh, 2014). More recently the experimental Ebola product, ZMapp containing three humanised mABs against the Ebola Zaire virus strain was produced in low-nicotine tobacco variety (*Nicotiana benthamiana*) which lacked plant-specific N-glycans (Olinger *et al.*, 2012). With further research into plant biology and recent significant progress in understanding the bottlenecks in protein expression, this model organism is now being considered as an alternative producer (T.K. Huang & McDonald, 2009). Indeed, the first plant cell culture recombinant protein drug is now on the market after approval from the FDA, being produced in carrot cells (Walsh, 2010b).

1.2 mRNA translation/protein synthesis and protein secretion in eukaryotes

1.2.1   Introduction to mRNA translation

Understanding the process of mRNA translation and how the different stages of this process are regulated is key to exploiting protein production in all organisms. mRNA translation is controlled at multiple stages; initiation, elongation and termination. At each stage, translation factors transiently associate with the ribosome to facilitate and control the process of polypeptide production. Although these steps are highly conserved between prokaryotes and eukaryotes, initiation is more complex in eukaryotes as they require at least 12 initiation factors compared to 3 in prokaryotes (Passmore *et al.*, 2007). Our understanding of the eukaryotic ribosome (which is larger) is also sparse compared to the prokaryote. Ribosomes are large macromolecules which consist of ribosomal RNA (rRNA) and 50-80 ribosomal proteins determined using X-ray crystallography and 3D cryo-EM (Spahn *et al.*, 2001). All ribosomes are composed of two subunits, the 60S subunit in *S.cerevisiae* is the largest consisting of three rRNA molecules (25S, 5.8S and 5S) with 46 proteins compared to the 40S subunit containing one rRNA chain (18S) and 33 proteins. There is variation between yeast and humans in the makeup of certain rRNA however the protein components are very similar (Jenner *et al.*, 2012).

1.2.1.1 Translation Initiation in Eukaryotes

There are important control mechanisms in place at initiation where the ribosome is recruited and assembled at the 5' region of the mRNA. This recruitment is undertaken by the ternary complex containing eIF2 and initiator Met-tRNA binding to the 40S subunit, aided by other factors forming the MFC (multifactor complex) forming 43S preinitiation complex (PIC) (see figure 1.3 for more detail)(Mead *et al.*, 2014). The PIC scans downstream to enter the P site (reading AUG) for complementarity to the anticodon of Met-tRNAi. eIF2-GDP and other eIFs are released and the large 60S joins forming the 80S initiator complex which is then ready to accept the suitable aminoacyl-tRNA into the A (aminoacyl) site to synthesise the first peptide bond (Sonenberg and Hinnebusch, 2009). This regulation allows for rapid, reversible and spatial control of gene expression which has been has been heavily studied in understanding this complex process (Jackson *et al.*, 2010).
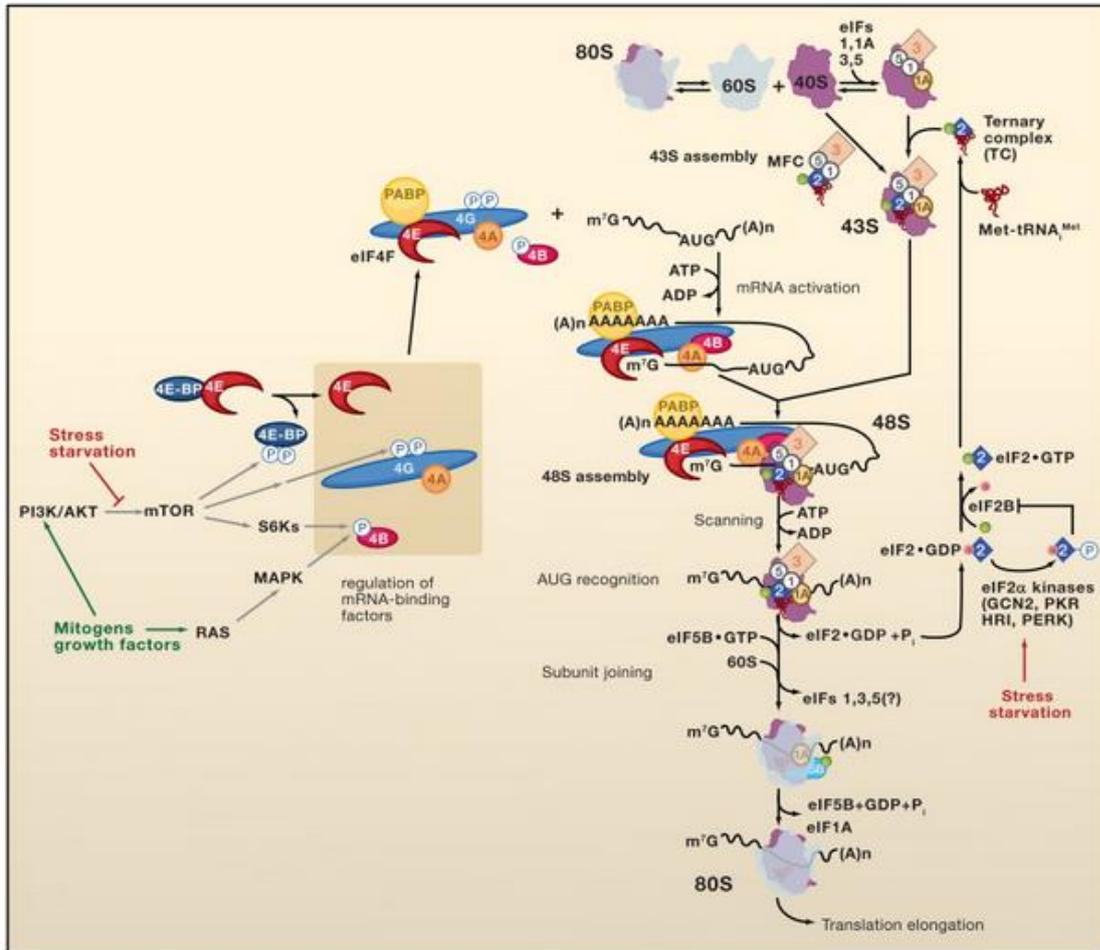
Figure 1.3 Schematic of eukaryotic cap-dependent translation initiation and its regulation. To assemble the 43S preinitiation complex (PIC), the 80s ribosome is dissociated by eIFs 1, 1A, 3 and 5 which bind the ternary complex (eIF2-GTP-Met-tRNA) and the 40S ribosomal subunit. eIF6 which binds to 60S is also important in ribosome dissociation(Sonenberg and Hinnebusch, 2009). The mRNA becomes activated and circularised by eIF4F (eIF4E-eIF4G-eIF4A) binding to the cap and PABP binding to the poly (A) tail. PABP binds to eIF-4G via it's C-terminal (Gorgoni and Gray, 2004). The 43S PIC binds the cap and scans for the leader for the AUG codon (an ATP dependent reaction) where there is a partial hydrolysis of eIF2-bound GTP to eIF2-GDP-Pi. The 43S binding is due to eIF3/eIF5 interacting with eIF4G/eIF4B. Pi and eIF2-GDP are released when the AUG site is recognised triggering the dissociation from 40S. 60S ribosome joins with release of other eIFs, catalysed by eIF5B-GDP. The hydrolyses of GTP triggers the release of eIF5B-GTP and eIF1A giving the final 80S complex ready for the elongation phase (Sonenberg and Hinnebusch, 2009).

1.2.1.2 Controlling initiation for efficient gene expression

Initiation is a rate limiting step where the complex of proteins eIF4F and PABP bound poly(A) tail both bind to the cap at the 5' end, which synergistically enhances initiation efficiency (Gingold and Pilpel, 2011). The association of PABP1 to eIF4G is important for enhanced affinity of eIF4G to the cap and PABP1 to the poly (A) tail. It is this interaction which promotes the PIC recruitment (Gorgoni and Gray,

2004). There have been extensive studies into the interaction of PABP with eIF4G. For example, there was evidence that overexpression of mutant eIF4G caused reduced poly (A)-mediated translation. Another group investigating PABP discovered that overexpression of the PABP-interacting protein (Paip-1) in mammalian cell lines has conferred a modest increase of reporter mRNAs (Wilkie *et al.*, 2003; Gingold and Pilpel, 2011). Once the PIC is recruited to the 5' end, scanning begins of the 40S ribosomal subunit until AUG recognition which is flanked by the short sequence known as the 'Kozak sequence' (Marilyn Kozak, 1986). Once AUG is recognised, scanning ceases along with the reaction of hydrolysis of GTP bound to eIF2 in the TC, a reaction which is irreversible. However, leaky scanning can occur when the AUG is in a suboptimal context where this can be bypassed and initiation begins at a downstream initiator methionine codon (Racine and Duncan, 2010).

Kozak proposed the scanning hypothesis where it was observed that additions of secondary structures at the 5'UTR could block translation (M Kozak, 1986; Hinnebusch, 2014). It was discovered that the shortening of the 5'UTR caused a negative effect on the efficiency of initiation beyond about 20 nucleotides. In yeast when investigating the expression of *PGK1,* the shortening on the 5'UTR lead to a reduction in translational efficiency (Hinnebusch, 2011). The optimal context for translation initiation in mammals is GCCRCCaugG (with a purine for R), where a strong context contains A at -3 and a G at +4. Since then further research has found that for animals, fungi, plants and protists, nucleotides -3A/G (crucial for improved initiation), -2A/c and +5C were common (Marilyn Kozak, 1986; Van Der Kelen *et al.*, 2009). Additionally, repetition of G or A are important for initiation (Nakagawa *et al.*, 2008; Van Der Kelen *et al.*, 2009). Tikole *et al* (2008) created a neural network approach to predict translation initiation sites in human mRNA sequences with a weak Kozak context (Tikole and Sankararamakrishnan, 2008).

1.2.1.3 Effect of stress and starvation on initiation

A number of signalling pathways control translation, for example in response to starvation, stress or growth factors (in mammals) (see figure 1.3). For the regulation of protein synthesis during environmental stresses, eIF2α is phosphorylated, inhibiting the formation of TC complex needed for the ribosome to bind to the start 34site of the mRNA. With further understanding of how this initiation factor is controlled and its effects on translation, engineering strategies can be used to enhance yield and quality of recombinant therapeutic proteins (Underhill *et al.*, 2005). During amino acid starvation in yeast, free tRNA'a which accumulate directly activate GCN2 (general control nonderepressible protein 2) by binding to the histidyl-tRNA synthetase (HisRS)-like domain. When GCN2 phosphorylates eIF2α, this causes low levels of TC as eIF2 becomes a competitive inhibitor of the GEF eIF2B (Magazinnik *et al.*, 2005).

In contrast to this, although phosphorylation of eIF2α causes inhibition of global translation, the translation of GCN4 is upregulated. When amino acids are plentiful, the ribosome translate uORF1 of GCN4, resume scanning and reacquire a TC before reaching the following 3 uORFs. After translation of the later uORFs, most ribosomes disassociate from the mRNA. However, when eIF2α is phosphorylated and there are lower levels of TC, ribosomes re-acquire the TC slowly after scanning through the remaining uORFs and reinitiate at the GCN4 ORF (Holcik and Sonenberg, 2005; Magazinnik *et al.*, 2005). Expression of this transcriptional activator causes expression of most amino acid biosynthesis enzymes necessary. In mammalian cells, the phosphorylation of eIF2α also triggers the translation of ATF4 (via a similar method to GCN4) containing two uORFs. This transcription factor targets genes such as ASN and CHOP. As in yeast where GCN2 phosphorylates eIF2 α, the other kinases HRI, PERK, PEK and PKR are also controlling the initiation factor (Gale Jr *et al.*, 2000).

Through understanding of the mechanisms behind the factors involved in translation and careful investigation into how these various factors are controlled, many novel ideas have been tested in improving rPP. Through the control of eIF2 phosphorylation, it has also been shown that when investigating the expression of a luciferase reporter, it expression was enhanced when simultaneously expressed it a mutated non-phosphorylatable eIF2αSer[51]Ala (Underhill *et al.*, 2003). Additionally, Underhill *et al.* (2005) proposed that as they found levels of GADD34 (activated by CHOP) correlated with eIF2α dephosphorylation levels and a recovery in protein synthesis, that GADD34 should be overexpressed in CHO cells. Another approach in prolonging protein synthesis was to overexpress p58 (which inhibits the kinases PKR and PERK), delaying eIF2α phosphorylation (Roobol *et al.*, 2015).

However, when it comes to controlling specific factors involved in translation, there are multiple pathways involved which have the potential to be exploited. For example, phosphorylation of factors such as 4E-BP and 4B depend on growth signals from pathways TOR (target of rapamycin) and MAPK, which are inactivated under certain circumstances such as starvation thereby directly effecting translation (Sonenberg and Hinnebusch, 2009), see more details on these pathways in sections 1.3.4.

1.2.1.4 Elongation and termination

This step requires elongation factors eEF1A and eEF1B for peptide chain elongation. eEF1A binds GTP and recruits aminoacyl-tRNAs to the A-site matching the codon at the site, where eEF1B acts as a GEF for eEF1A (Proud, 2007). This GEF is needed as upon codon-anticodon interaction GTP hydrolysis results in the eEF1A:GDP complex which needs to be exchanged preparing eEF1A for a new elongation cycle (Mansilla *et al.*, 2002). The peptidyl transferase centre catalyses formation of the peptide bond leaving a deacylated tRNA in the ribosomal P-site with the newly formed peptidyl-tRNA in the A-site.

eEF2 is also needed to catalyse the co-ordinated movement of the two tRNA molecules, the mRNA and conformational changes to the ribosome (Kaul *et al.*, 2011).

The mRNA contains a stop codon (UAA, UAG or UGA) which the ribosome encounters when elongation of the polypeptide is complete. At the point where the stop codon reaches the A-site, the ribosome releases the polypeptide. This process depends on the release factor eRF1 (*SUP45* in yeast) which causes peptide release by inducing hydrolysis of the peptidyl-tRNA:peptide bond (Merritt *et al.*, 2010; Blanchet *et al.*, 2015). eRF3 (*SUP35* in yeast) physically interacts with eRF1 depending on its hydrolysis of GTP although the molecular details of stop codon decoding are unclear (Merritt *et al.*, 2010).

1.2.1.5 Controlling the speed of translation elongation

Protein synthesis is mostly regulated at initiation, however there have been investigations into elongation and what parameters may slow down translation. The speed of translation depends on various factors such as the mRNA structure, abundance of translation factors, ribosome availability and the adaptation of the coding sequence to the cellular tRNA pool. The abundance of tRNA and codons of the gene will determine the speed of translation elongation as a selection of the appropriate tRNA matching the codon in the ribosomal A-site is needed (Tuller *et al.*, 2010). tRNA biogenesis therefore effects the speed of the decoding process impacting the speed of ribosome movement (Tarrant and von der Haar, 2014). Translation rates can slow due to the length of the open reading frame ORF or if it contains a stable RNA secondary structure which will cause pausing of elongation. In recombinant protein production there have been strategies towards improving translation by codon optimisation where the nucleotide sequence must be modified to optimise its codon preference without changing the amino acid sequence to suit the host (Mattanovich *et al.*, 2012). For example there is an inherent codon bias when producing protein as some transfer RNAs (tRNAs) are found in limited amounts; these are arginine, proline, isoleucine and leucine. One approach is to create a plasmid containing extra copies of the rare tRNAs, while another is to alter the gene with the majority of rare codons replaced by ones which are more frequent. An example is the gene *Gaussia* luciferase which was codon-optimised for efficient expression in mammalian cells (Tannous *et al.*, 2005). Aminoacylated tRNAs will interact with the A-site of a ribosome (which requires aminoacyl-tRNA synthases) with eEF1A and GTP. These aminoacylated tRNA's are classed into three groups, the first being non-cognate (non-complementary to codon), near cognate tRNA (with non-matching codon but limited complementarity) and cognate tRNA (with matching amino acid to the codon) (Tarrant and von der Haar, 2014). It is important to consider the trade-off between speed and accuracy as tRNA and decoding are the most error prone (Wohlgemuth *et al.*, 2011).

The frequency of ribosome binding to the mRNA needs to match the amount of the protein required by the cell, which is particularly important to consider when attempting to intrinsically enhance protein production (von der Haar *et al.*, 2004). A good availability of ribosomes is needed for faster translation and abundance of factors which control binding to form the 80S initiation complex (Mitarai *et al.*, 2008). For example fast-growing organisms such as yeast need a large abundance of ribosomes however possess very few free ribosomes during log phase (von der Haar, 2008), and the ribosomes that bind to the initiation region must transit efficiently for the next ribosome to start initiation (von der Haar, 2008; Tarrant and von der Haar, 2014). The speed of ribosome movement on mRNA can be reduced by the slow rate of peptidyl transfer reaction of certain amino acids (i.e glycine and proline), but also by the interactions between the nascent peptide and the ribosomal exit tunnel. Ribosome stalling can occur where a ribosome is already in front on the mRNA, therefore slowing movement (Mitarai *et al.*, 2008).

Although much work has been done investigating the speed of initiation and elongation, further optimisation to translation also includes removing unstable signal sequences, removing potential splicing sites and increasing the GC content which has been shown to increase expression (Zhu, 2012). Evidence has also shown that the rate of elongation has a significant impact of the quality of protein folding, where by partially inhibiting elongation (using inhibitors such as cycloheximide and emetine), this can correct for folding defects (Meriin *et al.*, 2012). The drug guanabenz which leads to the upregulation of eIF2 phosphorylation, had also caused improvement to folding, possibly by reducing the load on ER chaperones (Sherman and Qian, 2013). With our knowledge of various model systems (as discussed above) and their advantages as expression systems combined with our increasing knowledge on translation, recombinant protein secretion is continuously improving. Detail on the secretory pathway in eukaryotes and how protein folding affects quality of the protein is discussed below.

1.2.2 Secretion of protein

1.2.2.1 Introduction to the secretory pathway

In all eukaryotic cells, proteins destined for secretion or insertion into the plasma membrane, are transported into and through the endoplasmic reticulum (ER). The ER forms part of an ordered membranous network that comprises the secretory pathway (Schröder and Kaufman, 2005). Secretory proteins can be co-translated across the ER membrane as a consequence of an N-terminal signal peptide directing it to the ER post-translationally with the help of cytosolic molecular chaperones which belong to the Hsp70 and Hsp40 families. In *S.cerevisiae*, both the co-translational and post-translational pathways are equally important, compared to mammalian cells where the co-translational pathway is dominant (Zimmermann *et al.*, 2011).

In co-translation, a signal recognition particle (SRP) involved in ER targeting, binds to the signal peptide which is a ribosome-nascent chain complex causing translational elongation arrest. The SRP then transfers the complex to the ER (Idiris *et al.*, 2010). Once the ribosome docks to the ER membrane, the SRP binds to its receptor on the ER surface (SRP-receptor, SR). There is a transfer of the nascent polypeptide into a channel of the ER known as the Sec61 complex, which in turn relieves the elongation block. Due to this, the polypeptide is translocated across the ER lipid bilayer while it is being synthesised, preventing improper localisation in the cytosol (Kaufman, 2004; Vannuvel *et al.*, 2013) (see figure 1.4).

After entering the ER, folding and any assembly and processing, secretory proteins typically transit to the Golgi complex for further processing. This organelle was identified as membrane-enclosed flattened cisternae held as parallel stacks with tubular networks. Properly folded proteins are packaged in membrane bound vesicles and sent, to the cis-Golgi network which is the acceptor compartment and the trans-Golgi network responsible for sorting proteins to their next destination (Farhan and Rabouille, 2011). These vesicles selectively incorporate proteins from the ER and are targeted for the Golgi by coat protein complex II (COPII) (Gasser *et al.*, 2008)(figure 1.4). A retrograde transport pathway relies on the balance of COPII and COPI to recycle vesicle components, which involve the components activated Sar1 which recruits Sec23-Sec24 dimeric complex capturing cargo protein. An outer layer of the pre-budding complex is formed with Sec13-Sec31, and with the help of Sec16 forms a strong association with the ER membrane, fusing the vesicle to the cis-Golgi network (Farhan and Rabouille, 2011).

Within this organelle, proteins also undergo PTM (post-translational modifications) such as the biosynthesis of *O*-linked glycans and remodelling of *N*-linked oligosaccharides (more detail below) (Teasdale and Jackson, 1996; De Matteis and Luini, 2008). After protein processing, different cargo molecules are sorted and targeted for their final destinations such as the cell surface or early endosomes and vacuoles directly, or via the late endosome/mutivesicular body (MVB)(Bowers and Stevens, 2005). For trafficking of these proteins, sorting signals containing sequence motifs or structural determinants interact with recognition proteins, although PTM of the cargo has also been known to affect its sorting (De Matteis and Luini, 2008). Exocytosis of secretory proteins involves vesicles which dock to and fuse to the plasma membrane releasing the cargo outside the cell. The mutiprotein complex exocyst mediates this by interacting with v-SNAREs (Snc1/2 proteins in vesicles in yeast) and t-SNAREs (where the target membrane has Sso1/2 and Sec9) (Gasser *et al.*, 2008).
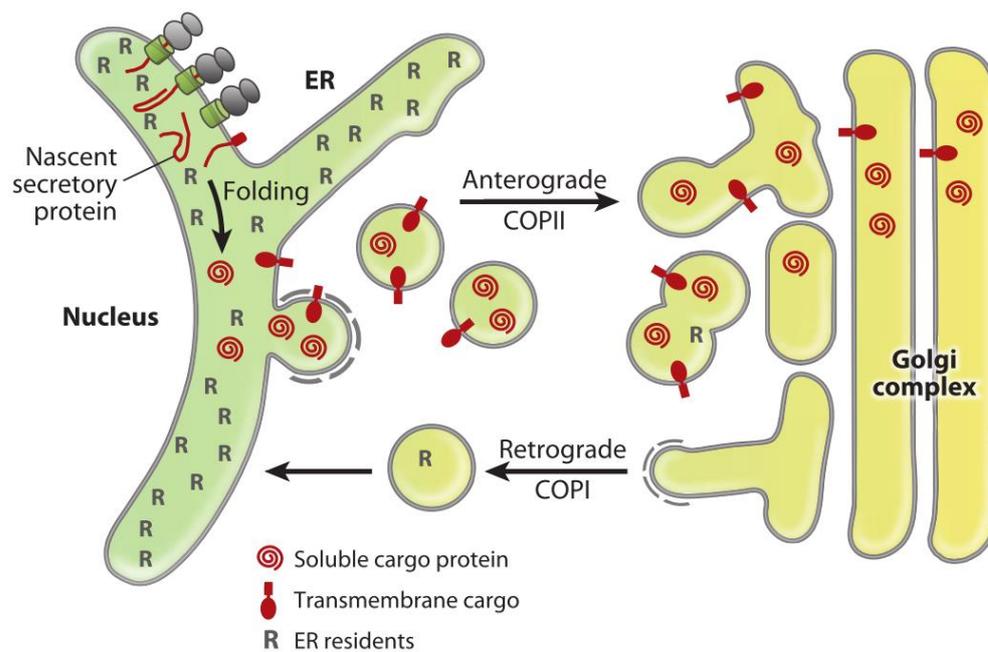
Figure 1.4 Transport of cargo between the ER and the Golgi. Soluble secretory cargo, as well as fully folded integral membrane cargo, are exported out of the ER by the coat protein complex II (COPII) transport vesicles and travels to the Golgi in an anterograde direction. To retrieve ER resident proteins such as chaperones and recycle vesicle components, COPI coats bud vesicles from the Golgi which are retrograde-directed (Dancourt and Barlowe, 2010).

1.2.2.2 Protein folding and quality control

The ER plays a role in the folding, sorting and delivery of proteins to the appropriate cellular destination as described above. With the help of chaperones, newly synthesised proteins are correctly folded and undergo PTMs (Bravo *et al.*, 2012). Proteins folding inside and outside the ER are governed by the same principles; and although the ER provides a suitable environment its physiochemical, chemical, and biochemical features can contribute to folding problems. Most PTMs are introduced via specific enzymatic steps and/or pathways. Some are more common than others for example glycosylation is common while AMPylation is rare. Certain PTMs are predominatly or exclusively associated with intracellular or extracellular proteins. Phosphorylation and ADP ribosylation are needed intracellularly whereas glycosylation, disulphide linkage formation and carboxylation are extracellular (Walsh, 2010a).

Glycosylation is the most complex PTM when it comes to therapeutic proteins (Walsh, 2010a). For protein folding, glycosylation increases the stability of glycoproteins due to the hydrophilic nature of carbohydrates. Due to their large hydrated volume, they also shield the attachment area from surrounding proteins acting as a chaperone and can interact with the peptide backbone, stabilising its conformation (Schröder and Kaufman, 2005). Initial glycosylation can occur during translocation which comes in two varieties in yeast, *N*-linked and *O*-linked. *N*-linked glycosylation in the ER is completed by oligosaccharyl transferase (OST) where a 14 sugar glycan tree is added to an asparagine residue. *O*-linked glycosylation however is catalysed by O-mannosyltransferases (PTMs) where a single mannose is transferred to the hydroxyl groups of serine and threonine of the peptide. Subsequent addition can occur in the Golgi (Hou *et al.*, 2012; Hakamata *et al.*, 2015).

The chaperone BiP (also known as GRP78 and Kar2) binds to hydrophobic areas of the peptide and plays a role in both protein translocation and in folding alongside other Hsp40 co-chaperones and Grp94 (which is not present in unicellular eukaryotes) (Brodsky and Skach, 2011) (see figure 1.5). Disulphide bond formations are catalysed by protein disulphide isomerases (PDI) which passes an electron from the disulphide bonds to FAD-dependent oxidases Ero1p and Erv2p (Schröder and Kaufman, 2005; Hou *et al.*, 2012). Disulphide bonds help to stabilize and maintain the tertiary structure and quaternary structure in multi-subunit proteins such as antibodies. As oxidation and reduction coexist in the ER, disequilibrium can cause ER stress as a reducing environment in the ER (as is in the cytosol) prevents the formation of disulphide bonds, while over-oxidation will stabilise non-native bonds causing misfolding (Margittai and Sitia, 2011).

After glycan attachment, glucosidase I (GlcI) and GlcII remove the first and second glucose consecutively, for high affinity to the proteins calnexin (CNX) and calreticulin (CRT). Where CNX is membrane bound and binds co-translationally, CRT is a soluble protein which will interacts with the protein after it is released by the ribosome and free in the ER lumen (Brodsky and Skach, 2011). At this point when the glycoprotein is either CNX or CRT bound, ERp57 will form disulphide bonds. If there is improper folding, the glycoprotein is reglucosylated by uridine diphosphate glucose:glycoprotein glycosyl transferase (UGGT) (Naidoo, 2009). The functions of these enzymes are summarised in table 1.1 below.

Table 1.1 Enzymes involved in protein processing and folding in the secretory pathway. Adapted from Naidoo (2009), Gasser et al. (2008) and Brodsky & Skach (2011).

Enzyme	Function
Immunoglobulin binding protein (BiP)/GRP78/Hsp5A	ER chaperone involved in protein folding and degradation. Part of HSP70 family
Glucose regulated protein 78 (GRP94)	Chaperone part of HSP90 family involved in protein folding
Protein disulphide isomerase (PDI)	Thiol-disulphide oxidoreductase - Folding and disulphide bond formation. Role in degradation.
Ero1	FAD-bound oxidoreductin 1 – accepts electrons from PDI in disulphide bond formation
ERp57	Thiol-disulphide oxidoreductase - Disulphide bond formation
Calreticulin (CRT)	Role in folding glycoproteins
Calnexin (CNX)	Role in folding glycoproteins (membrane bound) and quality control
UGGT	Reglucosylation of misfolded glycoproteins
Glucosidase I/II (Glc I/II)	Trims off terminal glucoses on glycans
oligosaccharyl transferase (OST)	Role in N-glycosylation of peptide
O-mannosyltransferases (PMT)	Role in O-glycosylation of peptide
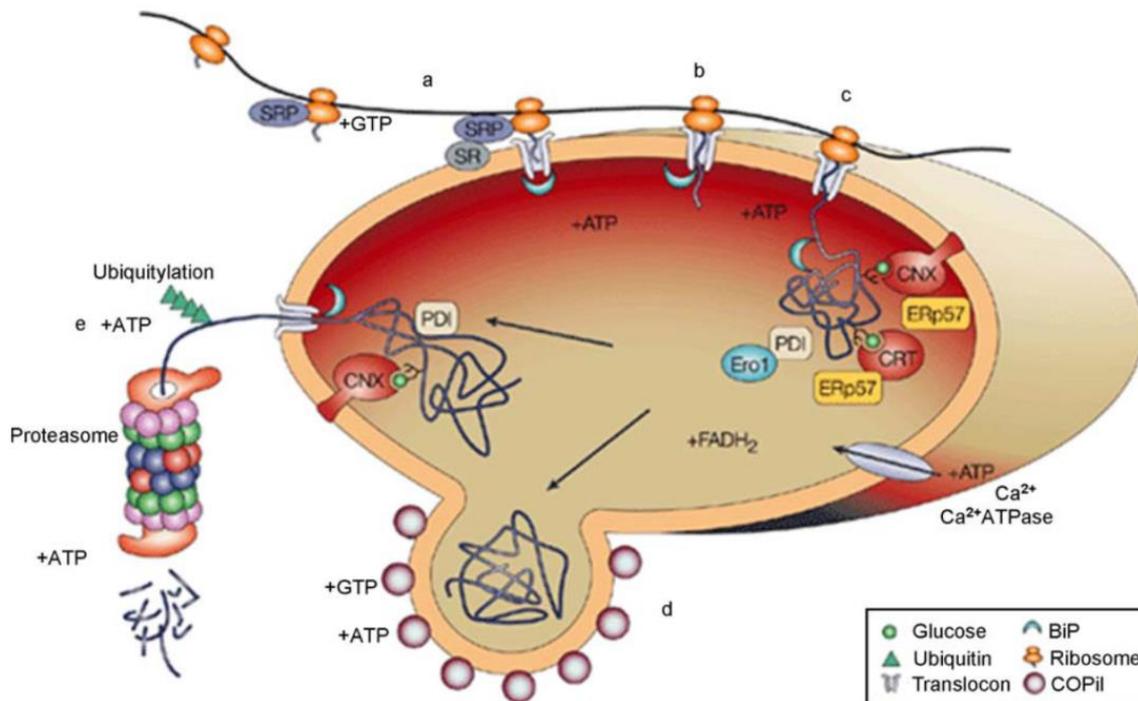
Figure 1.5 Simplified schematic of processes and chaperones involved in protein folding in the ER. (a) The signal peptide is recognised by the signal recognition particle (SRP) which associates with the ribosome when a protein is entering the secretary pathway. At this point the SRP binds to its receptor on the ER surface (SRP-receptor, SR). (b) BiP activity is involved as the growing peptide passes through the translocon. (c) Once the peptide enters the ER, chaperones BiP, calnexin (CNX) and calreticulin (CRT) act to fold the protein whereas disulphide-bond formation is formed in protein by the activity of Ero1 through the protein disulphide isomerase (PDI). ERp57 associates with CXN or CRT for access to the protein where bonds formed are modified. (d) COPII-coated vesicles are formed for the exit of properly folded proteins. (e) If proteins are misfolded, they can associate with CNX, PDI and BiP for retrotranslocation for ATP-dependent ubiquitylation and broken down by the proteome (Naidoo, 2009).

1.2.2.3 ER stress

Research has shown that processes within the ER actively monitor the folding status of its cargo. These systems communicate with other cellular compartments to elicit responses such as increased expression of genes involved in protein folding or indeed the induction of apoptosis. An example of this occurs if the folding capacity of the ER is exhausted, the normal physiological state of the ER is perturbed inducing the unfolded protein response (UPR) (Schröder and Kaufman, 2005).The UPR is involved in increasing the capacity of the secretory pathway by clearing misfolded proteins. Usually a portion of BiP is associated with immature proteins while the majority are associated with the ER transmembrane protein Ire1 preventing dimerization. However, with an increase in BiP bound-

misfolded proteins, Ire1 dimerizes and the cytoplasmic portion self-phosphorylates (Hou *et al.*, 2012). Now activated, this catalyses the splicing of the pre-mRNA HAC1/XBP1 (removing the inhibitory intron), thereby allowing translation of Hac1/XBP1 and its nuclear transport and transcriptional activation at promoters of unfolded response element (UPRE) (Buchberger *et al.*, 2010).

In yeast, UPR is only initiated via the IRE system, however in mammals there are two additional UPR transducers known as PERK and ATF6, which transiently inhibit new protein synthesis and reduce components that may enhance stress. PERK phosphorylates eIF2α globally attenuating protein synthesis by limiting mRNA translation initiation; however ATF4, GRP94 and BiP are translated efficiently increasing protein levels. Increased ATF4 leads to an increase in CHOP (a pro-apoptotic TF) (Bravo et al., 2012; Brodsky & Skach, 2011). ATF6 migrates to the Golgi where it is cleaved by proteases S1P and S2P, releasing a fragment into the cytosol that then migrates to the nucleus, activating transcription of BiP, GRP94 and PDI (Naidoo, 2009). To increase folding, as molecular chaperones are upregulated and recruited; the size of the ER increases diluting the unfolded protein load thus reducing stress on the ER (Schröder and Kaufman, 2005). In combination these 3 proteins initiate an ER-to-nucleus signalling cascades to improve cellular function and protect the cell from further stress (Wu *et al.*, 2007).

Due to adaptive signalling of the UPR, slowly folding or permanently unfolded proteins are targeted for proteasomal degradation through the ER-associated degradation (ERAD) complex (Ellgaard, 1999; Vannuvel *et al.*, 2013). Proteins targeted for degradation have two routes, the first being retrotranslocation into the cytosol and ubiquitination before proteasomal degradation (ERAD pathway) or autophagy where parts of the ER are targeted to lysosomes or vacuoles (although it is not well understood to what extent this is involved) (Schröder, 2008). Unfolded glycoproteins are mannose trimmed by ER mannosidase (Mns1) which is targeted for ERAD when recognised by homologous Yos9 (yeast) or OS-9/XTP3-B (mammalian) proteins. The chaperones BiP and PDI also associate with ERAD proteins along with E3 ubiquitin ligases such as carboxyl-terminus of Hsp70 interacting protein (CHIP) targeting chaperone substrates for degradation (Ballinger *et al.*, 1999; Buchberger *et al.*, 2010; Brodsky and Skach, 2011).

In mammalian cells these ER chaperones and folding enzymes have established a highly redundant system where individual members recognise their substrates differently, so if one chaperone fails, another will interact with an incompletely folded protein (Ellgaard, 1999; Braakman and Bulleid, 2011). ER stress can cause many knock on effects to the cells and the organism. In lower eukaryotes, vacuolar membranes are affected and assembly of the cell wall and function of the plasma hindered. In humans, mutation to a cargo receptor causes loss of secretion of specific proteins leading to

diseases such as haemophilia due to no blood coagulation factors V and VIII. Accumulation of folding-incompetent proteins which are resistant to proteasomal degradation can cause neurodegenerative diseases such as Parkinson's by disrupting ER function and activating apoptotic-signalling pathways causing cell death (Davis *et al.*, 1999; Schröder and Kaufman, 2005; Naidoo, 2009; Dancourt and Barlowe, 2010).

1.2.2.4 Secretion and processing of recombinant protein

To produce authentic therapeutic proteins such as monoclonal antibodies in mammalian cells, the protein is normally directed through the secretory pathway. This strategy generally achieves high yields and accurate protein folding (Jossé *et al.*, 2010). However, the over expression of rP can lead to protein aggregation within the ER system and associated proteotoxicity (Jossé *et al.*, 2010). As roles of genes and a better understanding of protein folding is unveiled, engineering of cells can be investigated in the hope of improve folding. It was expected that by increasing BiP levels, this would stimulate secretion levels because of an increase in folding capacity, and it has been previously reported that BiP transcription was induced from high levels of heterologous proteins expressed, however findings when this was undertaken were inconsistent (Robinson and Wittrup, 1995; Gasser *et al.*, 2008). In the fungus *A.niger,* it was discovered that a certain threshold of BiP overexpression was needed; similarly PDI overexpression once optimised improved plant sweet protein thaumatin production (Moralejo *et al.*, 2001; De Pourcq *et al.*, 2010). It may be that fine-tuned expression of these genes are required and that co-expression of genes such as chaperones may strongly depend on the properties of the target protein (Gasser *et al.*, 2008).

As discussed above, a signal sequence is needed to interact with the SRP which directs the peptide to the ER. A number of N-terminal sequences have been devolved for the use of host systems, for example the signal MFα1 from the mating pheromone α-factor is commonly used in yeast (as is used in chapter 3 of this thesis) (Rothblatt *et al.*, 1987; Idiris *et al.*, 2010). As an example, human IgG1 production in yeast has been engineered, increasing levels up to 180-fold over the wild type. As well as the use of MFα1, invertase (SUC2, hydrolyses of sucrose) and acid phosphatase (PHO5, phosphate metabolism) are other examples used in yeast (Chaudhuri *et al.*, 1992; Li *et al.*, 2007). In addition the viral K28 preprotoxin (pptox) signal peptide has been used as an example of how we can harness viral signal peptides in rP production (Idiris *et al.*, 2010) (more information on this in chapter 3). The information above is proof of how our understanding of the secretory pathway can be manipulated to our advantage for improving rP production at different points during peptide processing.

1.3 Cell signalling and its regulation

1.3.1 The actin binding protein cofilin and its regulation in yeast

1.3.1.1 The actin cytoskeleton

Actin is a highly dynamic protein/molecule and well conserved amongst eukaryotes. As well as being an essential part of the cytoskeleton of eukaryotes, it participates in a number of essential cellular processes including endocytosis and vesicle trafficking, the generation of cell morphology, polarity, motility and cell division (Dominguez and Holmes, 2011). Actin from the budding yeast *Saccharomyces cerevisiae* is encoded by a single gene, *ACT1* which encodes for a protein which has approximately 85% homology to the actin found within vertebrates (Aghamohammadzadeh and Ayscough, 2010). The high degree of conservation is shown in the alignments of the human and yeast actin shown in figure 1.7.

```
H. sapiens      IEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKMTQIMFETFNTP 60
S. cerevisiae   IEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPMNPKSNREKMTQIMFETFNVP 60
                ******************************************:***:************.*

H. sapiens      AMYVAIQAVLSLYASGRTTGIVMDSGDGVTHTVPIYEGYALPHAILRLDLAGRDLTDYLM 120
S. cerevisiae   AFYVSIQAVLSLYSSGRTTGIVLDSGDGVTHVVPIYAGFSLPHAILRIDLAGRDLTDYLM 120
                *:**:********:********:********.**** *::*******:************

H. sapiens      KILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKSYELPDGQVITI 180
S. cerevisiae   KILSERGYSFSTTAEREIVRDIKEKLCYVALDFEQEMQTAAQSSSIEKSYELPDGQVITI 180
                ***:******:***********************  ***.***:************

H. sapiens      GNERFRCPEALFQPSFLGMESCGIHETTFNSIMKCDVDIRKDLYANTVLSGGTTMYPGIA 240
S. cerevisiae   GNERFRAPEALFHPSVLGLESAGIDQTTYNSIMKCDVDVRKELYGNIVMSGGTTMFPGIA 240
                ******.*****:**.**:**.**:**:**:*********:**:**.* *:******:****

H. sapiens      DRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISKQEYDESGPSIV 300
S. cerevisiae   ERMQKEITALAPSSMKVKIIAPPERKYSVWIGGSILASLTTFQQMWISKQEYDESGPSIV 300
                :************:**:*********************:*****************

H. sapiens      HRKCF 305
S. cerevisiae   HHKCF 305
                *:***
```

Figure 1.7 Actin sequence in humans and *S.cereivisiae.* The sequences of actin in humans and *S.cerevisiae* were retrieved from the NCBI protein database. This shows their sequence alignment comparison performed on EBI-clustalW online tool illustrating how conserved actin is. An asterisks (*) represents a single fully conserved residue. A colon (:) represents conservation between groups of strongly similar properties. A Period (.) indicates conservation but with weakly similar properties and a gap indicates residues with no conservation. NCBI protein database address (http:www.ncbi.nlm.nih.gov/protein/) and EBI-clustalW online tool (hhtp://www.ebi.ac.uk/tools/msa/clustalw2/)

Actin has the ability to change between monomeric G-actin, and filamentous F-actin. Polymerisation of F-actin occurs by the addition of monomeric actin to form filaments, a process that is aided by a number of actin binding and regulatory proteins (Aghamohammadzadeh and Ayscough, 2010). The rapid addition of monomers at the fast growing or barbed end of filaments, coupled to the dissociation at the opposite, pointed end of filaments gives rise to the phenomena of actin filament treadmilling. Associated actin-binding proteins (ABPs) have various functions such as actin monomer sequestration, filament severing, filament cross linking and filament barbed-end and pointed end capping (Dominguez and Holmes, 2011). As actin is highly conserved, so are its components and functions which has lead yeast geneticists and cell biologists to use the experimental power of yeast for investigating the regulation of actin. The visualization of the cytoskeleton through the cell cycle is important in these studies. Rhodamine-phalloidin has been used to stain F-actin to reveal the actin-based cytoskeletal structures mentioned above (Amberg, 1998). Filamentous actin structures found in *S.cerevisiae* comprise of cortical actin patches, actin cables and actin-myosin contractile rings (Adams and Pringle, 1984; Aghamohammadzadeh and Ayscough, 2010).

In mammals, changes in the cytoskeleton result in processes such as initiation of neurite outgrowth, axonogenesis and axon pathfinding. The larger number of regulatory pathways that impinge on the ADF/cofilin family suggests that they play a central role in integration of many of these signalling cascades (Sarmiere and Bamburg, 2004).

Although is well known that actin has a central role in the cell, recent research suggests actin also acts as a biosensor of environmental perturbations, linking actin to signalling process in the cell (Smethurst *et al.*, 2013). For example, actin has a critical role in endocytosis which helps regulate cell surface composition ensuring the appropriate receptors and transporters are exposed which is important for survival (Aghamohammadzadeh and Ayscough, 2010). Another role for actin is by the movement of organelles such as the ER, mitochondria and peroxisomes transporting them throughout the cell via actin cables (Moseley and Goode, 2006; Aghamohammadzadeh and Ayscough, 2010). Actin has an additional role in protecting newly formed buds from accumulating damaged proteins from the mother cell and prevents aggregation of mitochondria, playing a protective role in yeast (Aguilaniu *et al.*, 2003; Liu *et al.*, 2010) . It has also been suggested that actin dynamics play a role in cell death and stress responses (Gourlay *et al.*, 2004; Kotiadis *et al.*, 2012). With these potential roles in cellular responses by actin to the cell environment it is not surprising that links can be found between actin and signalling pathways such as MAPK (in cell wall integrity),TOR signalling (via TORC2) and cAMP/PKA pathway in apoptosis (Schmidt *et al.*, 1996; Gourlay *et al.*, 2004; Levin, 2011).

1.3.1.2 Cofilin's primary role in the cell

Cofilin is a well characterised actin-binding protein which belongs to a group of small molecules (15-20 kDa), the actin depolymerising factor (ADF)/cofilin family. Unicellular organisms such as *S.cerevisiae* produce only one member of this family compared to metazoans which often have up to three different genes encoding ADF/Cofilin proteins (Bamburg and Wiggan, 2002; Aghamohammadzadeh and Ayscough, 2010). Mammals produce three members of the ADF/cofilin family, ADF (also known as destrin), cofilin-1 (non-muscle tissue) and cofilin-2 (in differentiated muscle) (Bamburg and Bernstein, 2010). These isoforms have similar, but not identical, biochemical activities (Van Troys *et al.*, 2008). In mice, knockout of the cofilin-1 gene is embryonic lethal, whereas the only consequence of ADF knockout in mice is postnatal blindness due to thickening of the cornea after about 4 weeks of birth (Bamburg and Bernstein, 2010).

Regulators of actin dynamics have various functions to regulate the ability for actin to polymerise and depolymerise. This includes those that are monomer binding, capping proteins, depolymerising proteins and filament stabilisers. An actin monomer-binding protein, profilin, has been found to interact with formins at sites of actin cable nucleation (Bertling *et al.*, 2007; Aghamohammadzadeh and Ayscough, 2010). Profilin catalyses the exchange of ADP for ATP, returning subunits to the pool of ATP-actin bound to profilin ready to elongate barbed ends as they become available but blocking binding to filament point ends (Pollard and Borisy, 2003; Smethurst *et al.*, 2013). Cofilin (Cof1) is a member of the ADF/cofilin family of actin binding proteins, which disassembles actin filaments at the pointed/minus end (depolymerisation end). It binds to both filaments and monomers and prefers to bind to ADP-G-actin (Bernstein and Bamburg, 2010).

1.3.1.3 Regulation of cofilin

The predominant enzyme that phosphorylates cofilin in mammalian cells is LIM kinase (LIMK) at serine 3. Although regulation of cofilin by phosphorylation has not been identified, alignment of cofilin in *S.cerevisiae* shows serine 4 (corresponding to serine 3 in vertebrates), and mutation of the serine site to glutamine creates a lethal phenotype (Lappalainen *et al.*, 1997; DesMarais *et al.*, 2005). There are two isoforms of this kinase. LIMK1, which is expressed in neural tissue as well as most others and growth cones of cultured neurons compared to LIMK2 which is ubiquitously expressed. LIMK1 is regulated by small GTPase Rac and Cdc42, but a downstream target of these proteins is PAK1, a protein kinase and is a direct regulator through phosphorylation of Thr508. LIMK2 is regulated by Cdc42 and Rho (via the protein kinase ROCK, a direct activator on thr505)(Amano *et al.*, 2001; Sumi *et al.*, 2001; Mizuno, 2013). Testicular protein kinase 1 (TESK1) is another kinase which is closely related to LIM-

kinases (50% amino acid identity). TESK1 also phosphorylates the cofilin proteins. Yet in contrast to LIM-kinases, TESK1 is not stimulated by either PAK or ROCK (Toshima *et al.*, 2001; Mizuno, 2013).

After phosphorylation, cofilin proteins are reactivated by dephosphorylation due to the binding of general phosphatases such as slingshot (SSH) and so have a counteractive effect to the AC(ADF/Cofilin) kinases (Niwa *et al.*, 2002; Bamburg and Wiggan, 2002; Van Troys *et al.*, 2008)(see figure 1.8). Another mode of regulating actin dynamics is the stabilisation of the inactive form of AC and the interactions with AC binding proteins by the 14-3-3 family. This is a family of proteins consisting of 7 isoforms. Once bound, the conformation of cofilin proteins is not affected upon phosphorylation. 14-3-3 proteins protect cofilin proteins from dephosphorylation by decreasing the availability of inactive AC, regulating the cellular phosphocofilin pool (Birkenfeld *et al.*, 2003; Han *et al.*, 2007).
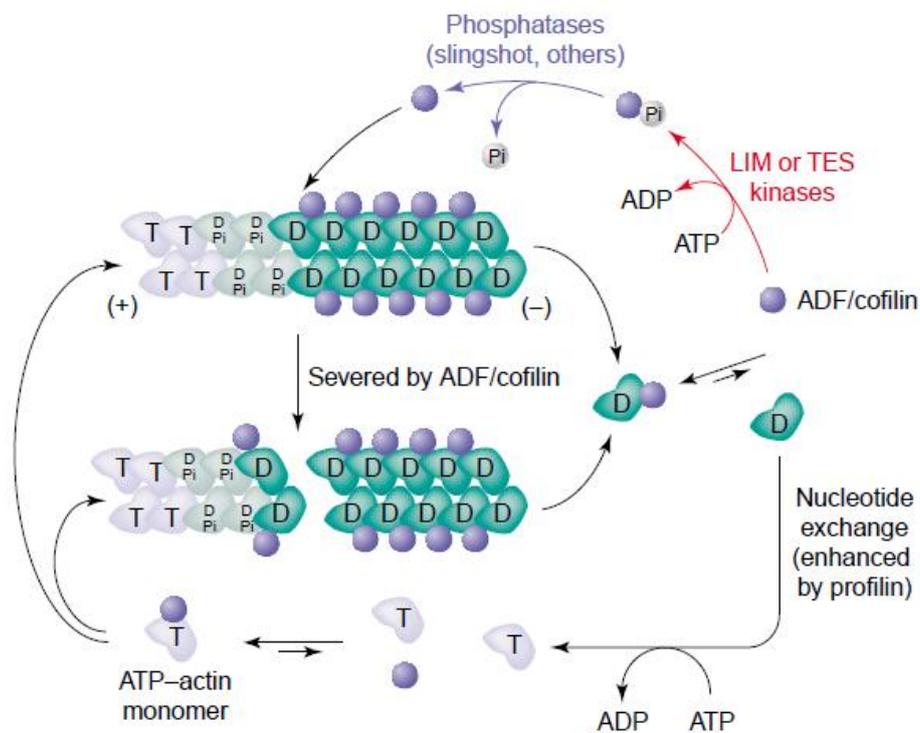
Figure 1.8: The activity and regulation of ADF/cofilin. These proteins bind to F-actin with the highest affinity for ADP-actin subunits. From the pointed end, ADF/cofilin proteins enhance the rate of subunit dissociation and sever filaments. This creates new pointed and barbed ends which can elongate or shorten. ADF/cofilin proteins are inactivated by phosphorylation of LIMK or TES. These can be reactivated by phosphatases. Abbreviations: D, ADP-actin; T, ATP-actin; Pi, inorganic phosphate (Bamburg and Wiggan, 2002).

1.3.1.4 Other roles of cofilin

In yeast *S.cerevisiae*, the identification of a cofilin homolog has opened new avenues for studying its function and as it is an essential gene, using yeast molecular genetics to identify cofilin residues important for cofilin-actin interactions (Lappalainen *et al.*, 1997), in addition discovering new roles of cofilin is possible. Previous studies had identified actin related activities of cofilin to be inhibited by phospholipids, in particular phosphatidylinositol 4,5-bisphosphate (PIP2). The PIP2 binding site was mapped on cofilin which overlaps with the area important for F-actin binding (Ojala *et al.*, 2001; DesMarais *et al.*, 2005). Although the function of PIP2 binding to cofilin is unknown, and as PIP2 can be found in multiple compartment of the cell, it has been suggested that this interaction could be an important for cytoskeletal dynamics and signalling (Kotiadis *et al.*, 2012).

1.3.1.5 Cofilin and the mitochondria

Actin has no nuclear localization sequence but there is evidence that cofilin chaperones actin to the nucleus. This may facilitate actin's functions in chromatin remodelling, formation of heterogeneous nuclear ribonucleoprotein complexes, and gene expression (Zheng, Han, Bernier, & Wen, 2009). Yet actin binding is not required for translocation of cofilin to the mitochondria or the release of cytochrome c. However, apoptosis is blocked by mutating the actin-binding domain. If actin dynamics are reduced, it is believed to cause a decrease in mitochondrial membrane potential as well as an increase in ROS levels (Chua *et al.*, 2003; Bernstein and Bamburg, 2010)

Mitochondria are membrane-enclosed organelles found in most eukaryotic cells needed for cellular respiration. Mitochondrial structure and function can be regulated through signalling pathways in response to environmental change, for example in nutrients or oxygen availability, (McBride *et al*, 2006). Oxidative phosphorylation is carried out in the inner membrane of mitochondria by a series of protein complexes known as the Electron Transport Chain (ETC). As some of the components of the ETC function as transmembrane proton carriers, a proton gradient is generated from the IMS to the matrix when electrons pass through the ETC. This proton gradient is necessary for conformational changes associated with ATP generation at the ATP synthase (Jarmuszkiewicz *et al.*, 2010). Mitochondria contain their own DNA (mtDNA), in *S.cerevisiae* these include mitochondrial-specific tRNA genes, two rRNA genes and genes encoding proteins involved in the electron transport chain. However, nuclear DNA also encodes for proteins that localize to the mitochondrion (Lipinski *et al.*, 2010).

Mitochondria are also crucial in decision points of cellular life or death. Apoptosis in yeast occurs via a simple intrinsic (mitochondrial) derived mechanism which is driven by the permeabilisation of the outer mitochondrial membrane (MOMP) and the production of ROS (Green and Kroemer, 2004; Klamt

et al., 2009). MOMP leads to the release of destructive proteins such as AIF (apoptosis-inducing factor) and endonuclease G which travel to the nucleus and degrade the genomic DNA (Li *et al.*, 2001). In addition, cytochrome c is released into the cytosol and mitochondrial fragmentation occurs. Prior to MOMP it is reported that mitochondrial hyperpolarisation occurs which is then followed by an oxidative burst, ROS production and the breakdown of mitochondrial membrane potential (Eisenberg *et al.*, 2007). In higher multicellular eukaryotes too little or too much apoptosis can result in disease (Mizushima *et al.*, 2008). It is believed that in yeast the control of cell death is also important for the management of multi-cell formations such as colonies or bio-films (Kuthan *et al.*, 2003). Recent work has shown that changes in the dynamic status of the actin cytoskeleton can act to modulate the apoptotic response to yeast, plants and animals (Franklin-Tong and Gourlay, 2008)**.**

Communication between organelles is important throughout the cell; signalling between ER and mitochondria in yeast is acheived through the physical connection of the protein complex referred to as ERMES (ER-mitochondria encounter structure)(Kornmann *et al.*, 2009). It is believed that the ERMES may be responsible for mitochondrial membrane biogenesis, genome replication, Ca^{2+} signalling and protein import; all regulating mitochondrial and cell physiology (Kornmann and Walter, 2010; Murley *et al.*, 2013). Ca^{2+} signalling in particular is important for modulating mitochondrial bioenergetics, influencing metabolism and survival.

During ER stress such as that experienced due to the accumulation of misfolded proteins, this leads to the triggering of the UPR to restore folding capacity as described in section 1.2.2.3 above. This enhances mitochondrial bioenergetics and ATP production, however prolonged UPR signalling from persistent stress will cause mitochondrial collapse, triggering cell death by apoptosis (Liu *et al.*, 1996; Bravo *et al.*, 2012). Although mitochondria can also buffer Ca^{2+} in the cytosol, preventing cytotoxic effects, it will eventually cause an opening of the permeabilization transition pore, releasing cytochrome c leading to apoptosis (Pinton *et al.*, 2008; Denton, 2009; Kornmann and Walter, 2010).

1.3.1.6 Cofilin's interaction with signal transduction and stress response pathways
Cofilin is an essential gene in eukaryotes and plays an important role in the control of a number of signalling pathways. It is believed that there is an integration of cytoskeletal activity with stress signalling. Previous and unpublished data from within the Gourlay lab suggests that in yeast cofilin may act as a regulator of three major networks which are controlled by TOR (Target of Rapamycin), Ras and MAP (mitogen-activated protein) kinase signal transduction cascades. More on these pathways are detailed below.

It has been determined that in CHO cells, transient knockdown of cofilin enhanced productivity of human secreted alkaline phosphatase (SEAP) by up to 80% suggesting that modulation of this protein

influences cellular properties that impact upon rP production. Cofilin was identified as a key protein through transcriptome and proteome profiling of cell lines which identified cytoskeletal proteins as new genetic targets (Hammond and Lee, 2012). As cytoskeletal proteins play a role in a large number of cellular processes such as cell cycle progression, secretory vehicle transport and metabolism these may be key targets for engineering strategies. Other research in mammalian cells has also suggested that phosphorylated cofilin might control phospholipase D (PLD2) which affects vesicular trafficking and therefore secretion (Han *et al.*, 2007). However this is all through the roles of cofilin in actin binding. Roles outside of cofilin's actin binding region have highlighted how mutations to this protein cause increased mitochondrial biogenesis, and as it is known that ER stress leads to changes in mitochondrial function, it is possible that these mutants may have better protection (with enhanced mitochondrial function) against stress caused from rP production (Kotiadis *et al.*, 2012; Curwin *et al.*, 2012). Preliminary data from the Gourlay lab also suggests that proteotoxic effects from rPP leads to loss of mitochondrial function. This evidence points towards cofilin acting as a homeostatic sensor outside its role in actin binding where it can act as a signalling molecule under stress, altering mitochondrial function. In the work reported here it was envisaged to investigate how other stress signalling pathways interact with this as there is already evidence from the Gourlay lab that cofilin mutant alleles have altered RAS, MAPK and TOR signalling. These signalling pathways are introduced in detail below.

1.3.2   Ras/cAMP/PKA pathway

Ras and cyclic AMP (cAMP) signalling in yeast coordinates cell growth and proliferation with nutritional sensing. During the exponential growth phase, RAS, a small GTPase is activated and promotes the upregulation of cAMP production from adenylate cyclase. This in turn activates three protein kinase A (PKA) subunits which act to downregulate stress tolerance, and promote cell cycle progression (Cazzaniga *et al.*, 2008). It has been demonstrated that a decrease in actin dynamics leads to ROS generation due to abnormal mitochondrial functionality. This was the first time a link had been established between actin dynamics, mitochondrial function and cellular health (Gourlay *et al.*, 2004)(Kotiadis *et al.*, 2012). Subsequent research found that this link was through Ras/cAMP/PKA signalling, regulating ROS production and cell fate (Campbell W Gourlay and Ayscough, 2005). Work from the Gourlay lab has provided evidence that cofilin mutations that stabilise F-actin also trigger hyperactivation of Ras leading to loss of mitochondrial function and apoptosis (Kotiadis *et al.*, 2012).

1.3.3   MAPK signalling and its role in stress signalling

MAPK are serine-threonine protein kinases which are activated by a range of stimuli such as cytokines, growth factors, neurotransmitters, hormones, cellular stress and cell adherence being among the most widely characterised (Widmann and Gibson, 1999; Cargnello and Roux, 2011). There are four MAPK pathways in *S.cerevisiae* involved in mating (Fus3), filamentous and invasive growth (Kss1), high osmolarity (Hog1) and cell wall integrity (Slt2/Mpk1) pathways (Molina *et al.*, 2010).
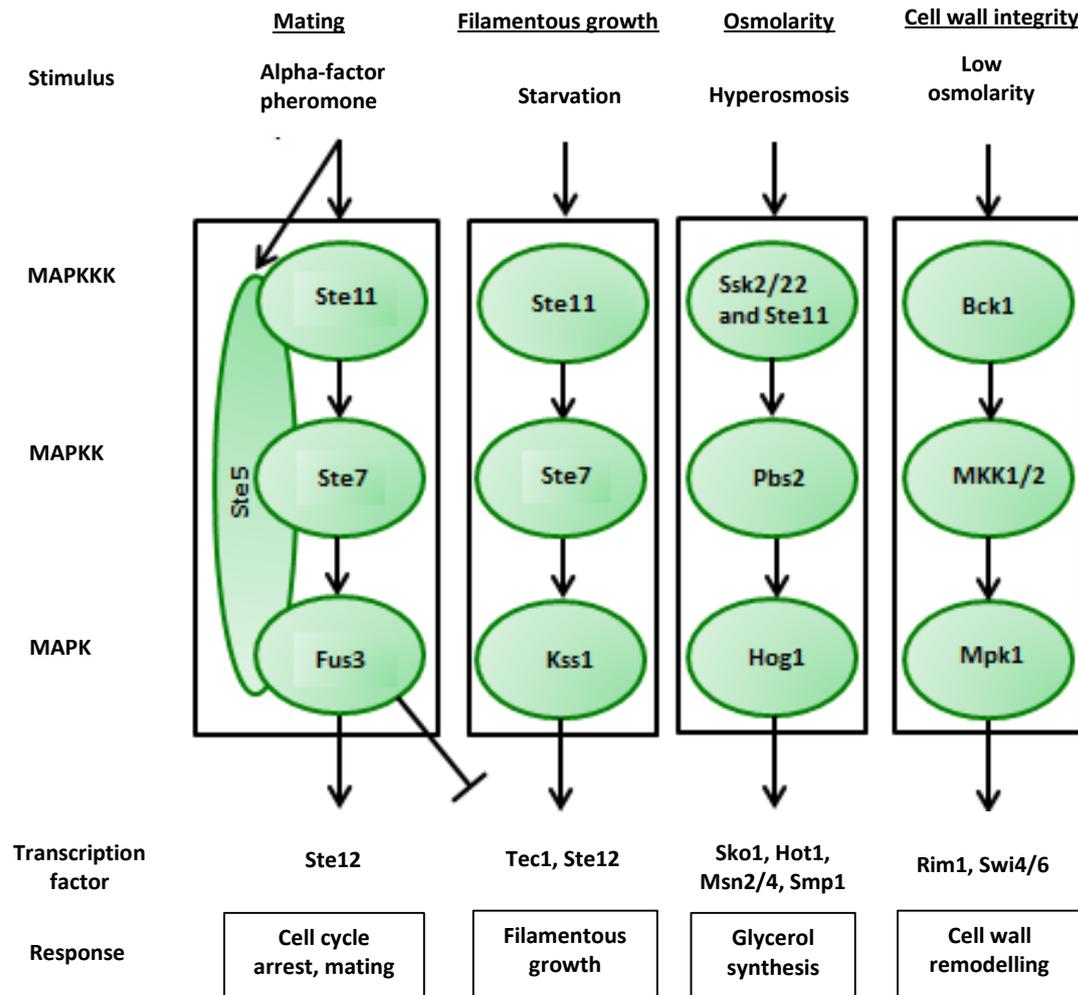
Figure 1.9 An overview of the three-compartment module of the 4 MAPK pathways in yeast. The diagram indicates stimulus which trigger these pathways and the response to these signals. Ste5 acts as a scaffold to Ste11, Ste7 and Fus3and promotes Ste7→ Fus3 signalling. A MAPKKK has not been identified during meiosis. Adapted from (Saito, 2010).

The MAPK cascades consist of three protein kinases that act sequentially and which are conserved from yeast to higher eukaryotes. Components of this cascade have a highly conserved molecular structure; therefore molecular mechanisms are in place to allow for the selective activation of each pathway. This specificity is conferred by the use of specific binding motifs that determine sequential physical interactions (Martín *et al.*, 2005; Molina *et al.*, 2010). This set of three sequentially acting

protein kinases starts with an activated MAPK kinase kinase (MAPKKK) which activates a MAPK kinase (MAPKK); this in turn activates a MAPK. The MAPK kinase cascade is initiated by phosphorylating MAPKKK by upstream events including processes such as occupancy of receptors coupled to heterotrimeric G proteins by their cognate agonists and/or the binding of the appropriate ligands to other classes of receptors that stimulate production of activated monomeric G-proteins (Chen and Thorner, 2007).

MAPK signalling pathways use overlapping sets of signalling components, the MAPKKK Ste11 is found in three out of the five signalling pathways. Due to insulation mechanisms, cross talk is avoided between pathways with shared components. One is through interactions with their substrates through docking domains, enhancing their fidelity and efficiency of action, these docking sites are short amino acid stretches (Sharrocks *et al.*, 2000; Saito, 2010). However, there is evidence that indicates that a coordinated interaction between these pathways is required. An example where this applies is when there is polarized growth in pseudohyphal development and mating. This requires the control by mating and filamentation signalling pathways as well as cell wall remodelling; therefore coordination is necessary between the CWI and other MAPK pathways (Rodríguez-Peña *et al.*, 2010).

1.3.3.1 MAPK in yeast mating and filamentous growth

During mating, the pheromone MAPK pathway is activated by cell–type specific mating pheromones. Yeast cells exist as either diploid or in haploid form, there are two sexual phenotypes. In *S.cerevisiae* during mating to create the diploid form, cells are controlled by the a or α pheromones which are controlled by the expression of a set of genes involved in mating (Madhani and Fink, 1998; Ydenberg and Rose, 2008). Pheromones bind to their receptors which are coupled to a heterotrimeric G protein which is activated leading to the dissociation of the βγ-complex from the α-GTP, this then stimulates the mating pathway. This βγ-complex contains Ste4 and Ste8 which activates Ste20, signalling to the MAPK module (Ste11→Ste7→Fus3) (Widmann and Gibson, 1999; Kholodenko and Birtwistle, 2009). The kinase Ste5 acts as scaffold protein, binding to Ste11, Ste7 and Fus3, changing the affinity of Fus3 as it acts as a substrate co-catalyst. Once Fus3 (MAPK) is activated, it signals to transcription factor Ste12 and temporarily arrests the cell cycle in G1 phase (Oehlen *et al.*, 1996; Saito, 2010; Wong Sak Hoi and Dumas, 2010). This is done by Ste12 inducing Far1 transcription as the cells must not grow. This induces remodelling of the cytoskeleton and the cell wall causing the cell to fuse with the mating partner (Tedford *et al.*, 1997; Widmann and Gibson, 1999; Wong Sak Hoi and Dumas, 2010). Mating in the fission yeast, *S.pombe,* also involves homologs of the yeast MAPK module but is controlled by different upstream regulators.

Filamentous growth (FG) is where cells become elongated, and mother and daughter cells remain attached to each other. This forms filaments of cells called pseudohyphae; cells go through this developmental change when there are poor nutritional conditions. Therefore when carbon or nitrogen is limited in the environment, this stimulates the FG MAPK pathway (Ste11→ Ste7 → Kss1) allowing sessile cells to seek out scarce nutritional resources. Kss1 controls cell adhesion, cell elongation as well as the reorganization of cell polarity through the activation of transcription factors Ste12 and Tec1 (Madhani and Fink, 1997; Bardwell *et al.*, 1998; Martín *et al.*, 2005).

The pheromone and FG pathway share Ste11 (the MAPKKK) and Ste7 signalling (the MAPKK). Yet pheromone activated Ste7 only activates Fus3 (MAPK), and Ste7 signalling induced by starvation only induces Kss1. Pheromone signalling activates both Fus3 and Kss1, but this is under tight control. Kss1 is controlled transiently where Fus3 is only activated when pheromone concentrations are above a certain threshold (Ydenberg and Rose, 2008; Saito, 2010). Therefore when Kss1 is activated, the FG pathway induces cell elongation, helping the cell to reach mating partners.

Ste12 is phosphorylated by Fus3 in pheromone response and Kss1 during filamentous growth. Ste12 is also regulated by Dig1 and Dig2 which block its activity (Bardwell *et al.*, 1998; Engelberg *et al.*, 2014) which are controlled by Fus3 and Kss1 (Wong Sak Hoi and Dumas, 2010). The way in which Ste12 is able to control two pathways is through different MAPKs and by binding to a DNA element called the PRE (pheromone response element) as a homomulitimer or as a heteromultimer with Mcm1, a protein which binds to the P box. This response to pheromone activates mating genes (Madhani and Fink, 1998; Bao *et al.*, 2004; Su *et al.*, 2010a). For filamentous growth during starvation, Tec1 binds with Ste12 which associates with the filamentous response element (FRE) which is a combination of the motif PRE and TCS (TEA/ATTS consensus sequence). However, some filamentous genes do not contain PRE motifs so regulation may be due to TCS. This results in the activation of genes needed for filamentation (Wong Sak Hoi and Dumas, 2010).

1.3.4 TOR signalling and its regulation in protein production

1.3.4.1 What is the role of TOR signalling?
Another major signalling pathway is TOR (target of rapamycin) which is involved in nutrient sensing, and if inhibited by the drug rapamycin or poor nutrient sources leads to prolonging of cell division and reduced cell size. The role of TOR in nutrient control of cell size is well conserved (Petersen, 2009).

Like other eukaryotes, *Saccharomyces cerevisiae* contains two TOR complexes, TORC1 and TORC2, but only TORC1 is rapamycin sensitive. In yeast, treatment with rapamycin induces phenotypic changes, for example, cell cycle arrest and entry into G0 phase, accumulation of the reserve carbohydrate glycogen and the stress protectant trehalose, general downregulation of protein synthesis, upregulation of stress response genes, autophagy and alterations in nitrogen and carbon sources.

Studies from the late 1980's led to the identification of the TORC1 binding protein FKBP12 (encoded by the FRP1 gene) in budding yeast. FKB12 was subsequently shown to be the target of rapamycin and to be required for its toxic, anti-proliferation action. Rapamycin does not therefore directly inhibit TOR kinase activity, but blocks interactions with regulatory proteins causing a conformational change or through steric hindrance that prevents function of this essential pathway (Heitman *et al.*, 1991; Fingar and Blenis, 2004; Hands *et al.*, 2009). This drug is a bacterial macrolide and is used as an antitumor and immunosuppressant drug by limiting cell growth (Barbet *et al.*, 1996; Claudio De Virgilio and Loewith, 2006; Jacinto and Lorberg, 2008). TORC2 is less well characterised in comparison and is thought to regulate the special aspects of growth including actin polarisation (Galdieri *et al.*, 2010). In mammalian cells, TOR is highly conserved with both TOR complexes but with a single gene mTOR (see figure 1.10 below).
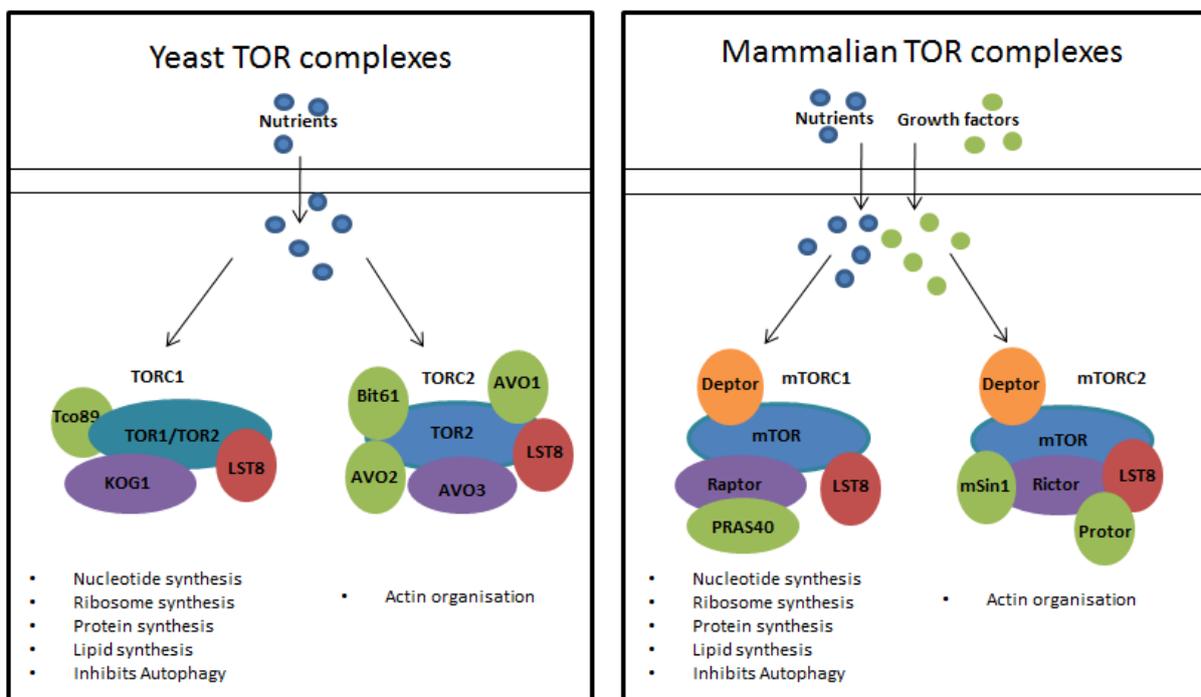
Figure 1.10 Comparison of the TOR complexes in yeast and mammalian cells. In yeast TORC1 has either Tor1 or Tor2 and the components Tco89, Kog1 and LST8. The TOR complexes sense nutrient availability, particularly leucine. Mammalian cells on the other hand have only one TOR gene, mTOR with Raptor, mLst8, Deptor and PRAS40. This complex and mTORC1 sense nutrients and growth factors in the environment. Adapted from Inoki et al., (2005); Jewell, Russell, & Guan, (2013).

In the TORC1 complex, mammalian cells contain raptor (KOG1 in yeast), which interacts with mTOR and is also believed to be sensitive to rapamycin and nutrients. This protein serves as a scaffold to present substrates to mTOR. As in mammals, KOG1 in budding yeast is an essential gene known to contain four internal heat repeats and seven C-terminal WD40 repeats. Lst8 is made up entirely of seven WD40 repeats and is found in both complexes in mammals and budding yeast. It is believed to act as a chaperone and is required for amino acid permease transport of Gap1 from the Golgi to the cell surface. Lst8 works by binding to the kinase domain of mTOR to stimulate mTOR kinase activity and its knockdown leads to defects in actin cytoskeleton reorganisation, indicating it also functions in mTORC2 (Jacinto and Lorberg, 2008). In yeast, TORC2 also contains AVO1, AVO2, AVO3 and BIT61. AVO1 and AVO3 are required for TORC2 integrity; AVO2 is nonessential and may recruit substrates to TOR2 (Wullschleger *et al.*, 2006). TORC1 also contains the subunit TCO89 a nonessential protein which was found to block glycerol uptake during osmotic stress when mutated. When Tco89 expression is knocked out/down, cells became hypersensitive to rapamycin suggesting a positive function in TORC1.

1.3.4.2 Upstream and downstream regulators of TORC1

In order for cells and organisms to integrate information from the environment with TORC1 and TORC2 signalling, these stimuli are detected by various upstream regulators of cell growth. In mammalian cells, stimuli such as growth factors stimulate AKT which phosphorylates tuberous sclerosis complex (TSC). TSC is comprised of TSC1 and TSC2 which together act as a GTPase-activating protein (GAP) for the small GTPase Rheb (RAS homologue enriched in brain) so converting Rheb into its inactive state (GDP bound). Therefore growth factors inhibit TSC, promote Rheb which in turn activates mTORC1 kinase activity. When glucose or intracellular energy (low ATP: ADP ratio) is low, this sensing inhibits mTORC1 directly by AMP-activated protein kinase (AMPK) which phosphorylates RAPTOR, and indirectly through TSC2. AMPK can also phosphorylate TSC2, reducing mTORC1 activity. TOR also balances energy metabolism/requirements by controlling mitochondrial genes. PGC1-α, a nuclear cofactor, plays a key role in mitochondrial biogenesis and oxidative metabolism, by directly altering its physical interaction with another transcription factor, yin-yang 1 (YY1) (Cunningham *et al.*, 2007).

The mechanisms by which amino acids are sensed and how this information is transmitted to mTORC1 in mammalian cells is less clear. The amino acids Leu, Gln and Arg have been implicated in the activation of mTORC1, yet it is not known for certain where the amino acids are first detected (Jewell *et al.*, 2013). In *S.cerevisiae*, nitrogen starvation and rapamcyin treatment cause a very similar responses suggesting TORC1 is regulated by the abundance of nitrogen. This nutrient signalling

cascade upstream of TORC1 at the vacuole (lysosome in mammals) where it is sensed by the EGO protein complex (Claudio De Virgilio and Loewith, 2006).

In yeast, TOR signalling may also mediate some of the responses to glucose and almost certainly exhibits cross talk with the Ras/cAMP pathway (Bertram *et al.*, 2002; Kuranda *et al.*, 2006). During rapamycin treatment, the transcript levels of several genes induced by glucose decrease and a number of glucose-repressed genes are expressed to higher levels. It has been reported that 14-3-3 proteins (Bmh1/Bmh2) in the presence of glucose, interact with Yak1 (cAMP/PKA pathway) which is phosphorylated by PKA. When Yak1 is activated it phosphorylates Crf1, a transcriptional inhibitor of ribosomal genes (Moriya *et al.*, 2001; Gancedo, 2008).

TORC1 positively regulates protein synthesis by controlling translation initiation, expression and assembly of the translation machinery. It also has a role in mRNA turnover and regulates the activity of high affinity amino acid permeases such as NPR1 (seen with rapamycin treatment). eIF4E-binding proteins (4E-BPs) are prevented from binding and inhibiting eIF4E by phosphorylation through mTORC1 for protein synthesis to occur (Sonenberg and Hinnebusch, 2009). TORC1 directly and indirectly controls protein synthesis by phosphorylating eIF4G, but also phosphorylates the major kinase Sch9 (S6K in mammals) which is then able to phosphorylate eIF4B required for its interaction with eIF3 during initiation (see figure 1.3 earlier in chapter) (Proud, 2007). During elongation, TORC1 (through S6K) keep eEF2K inactive, preventing phosphorylation of eEF2, yet when there is loss of TORC1 signalling, eEF2 becomes phosphorylated, slowing elongation (Proud, 2009).

TOR is an important cell size regulator and increases translation of mRNA transcripts coding for ribosomal proteins and other translational regulators. In yeast it was found that mutation in *Tap42* inhibited polyribosome formation, and *TPD3* (a subunit of PP2A) and *SIT4* function upstream of RNA pol III and pol II respectively (van Zyl *et al.*, 1992; Inoki *et al.*, 2005). Several factors regulate ribosomal protein gene expression through TORC1. In yeast, FHL1 for example binds to ribosomal protein promoters, when CRF1 forms a complex, expression is suppressed. When FHL1 has formed a complex with IFH1, expression of ribosomal protein genes are still stimulated. In the absence of these genes, SFP1, a transcription factor also regulates ribosomal protein genes. It is also known that TOCR1 controls mRNA turnover as both nutrient limitation and rapamycin treatment result in enhanced turnover of some but not all mRNAs (Martin *et al.*, 2004; C De Virgilio and Loewith, 2006a).
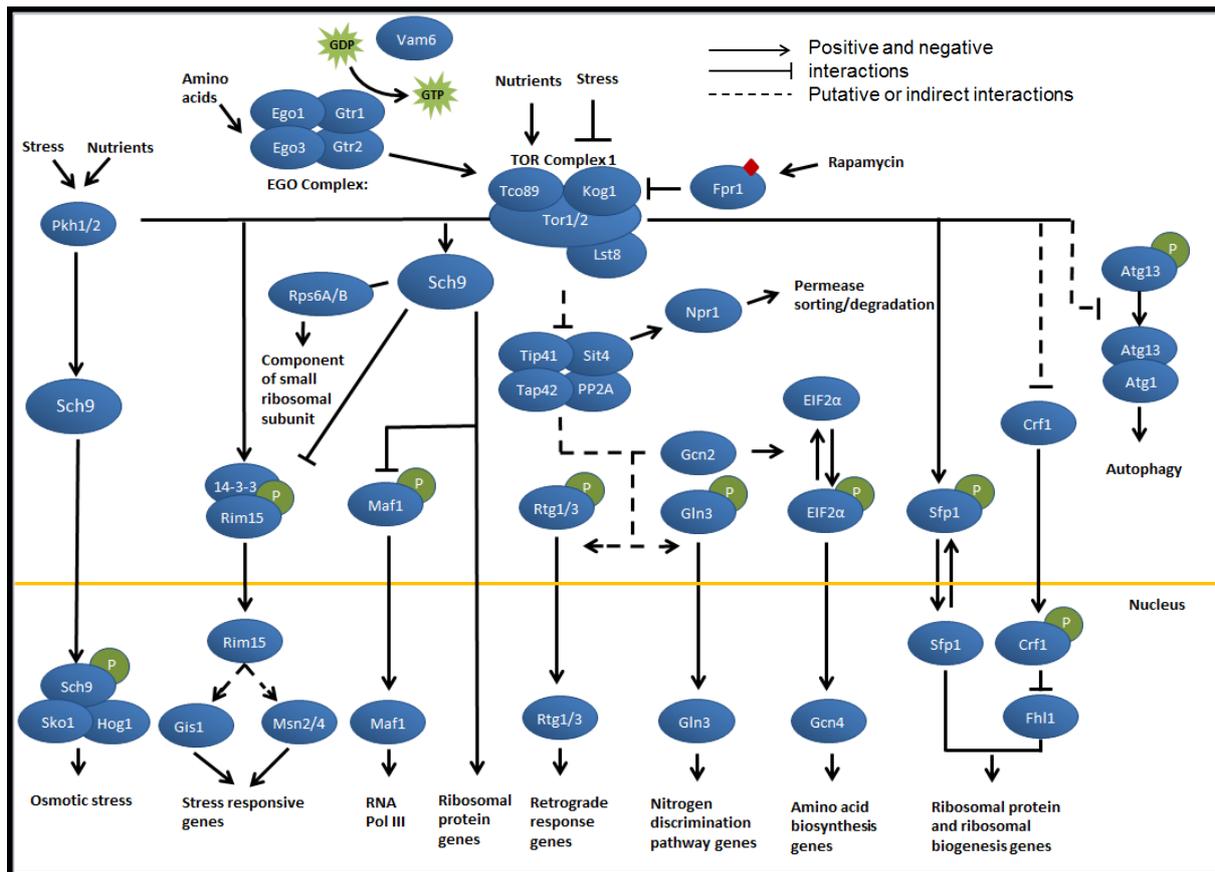
Figure 1.11 Simplified TORC1 pathway in yeast. Upstream and downstream regulators of TORC1 can be found in the cytoplasm and nucleus. Stimuli such as nutrients and amino acid signal through TORC1 to other kinases leading to changes in transcription and translation. The green P indicates phosphorylation. Key above shows arrows representing interactions. Adapted from Smets et al., 2010.

1.3.4.2.1 Sch9

The protein kinase Sch9 (in yeast) is an important component of the TOR signalling pathway and plays a central role in nutrient-mediated signalling. Sch9 functions in both the cytoplasm and the nucleus. Loss of this kinase results in slow growth, reduced cell size and small colony formation. Overexpression of a component of the PKA pathway (CDC25, maintaining cAMP levels) suppresses these phenotypes (Toda *et al.*, 1988; Hartley *et al.*, 1994; Smets *et al.*, 2010). Sch9p protein kinase is thought to work in parallel to RAS/cAMP/PKA signalling as they regulate common genes required for entry into the G0 phase regulated by Rim15 (Rohde *et al.*, 2008). The regulation of Rim15 by phosphorylation of Sch9 was found to be dependent on TORC1 signalling as expected. Sch9 also independently regulates the effectors Msn2/4 and Gis1 explaining why Sch9 and TORC1 can have opposed effects to the expression of stress responsive genes (Urban *et al.*, 2007; Wei *et al.*, 2008; Smets *et al.*, 2010) (see figure 1.11).

It is thought that Sch9 is subject to negative feedback control as it negatively regulates Sfp1 (transcriptional activator of ribosomal biogenesis) which itself is activated by TORC1. Sch9p has also been implicated as a transcriptional regulator and is involved in osmotic stress associating with Sko1 and Hog1 from the HOG pathway (Pascual-Ahuir and Proft, 2007; Smets *et al.*, 2010).

Sch9 belongs to the AGC kinase (protein kinase A/protein kinase G/protein kinase C) family where TOR targets the conserved hydrophobic motif located at the C-terminal tail. It was originally believed that protein kinase B (PKB/AKT) was the mammalian homologue due to its pronounced homology at the catalytic domain and C-terminus. Both PKB and Sch9 are activated by the TORC1 and the kinase PDK1 (orthologs Pkh1 and Pkh2 in yeast). However, other work has shown that the TORC1 substrate S6K1 is more closely related to Sch9 with S6K1 also being regulated by TORC1 and performs similar functions. In yeast Sch9 is able to phosphorylate Rps6 (mammalian RPS6) regulating translation initiation as well as ribosomal biogenesis (Urban *et al.*, 2007).

1.3.4.3 Nutritional sensing of TORC1 via the EGO complex
In yeast amino acids activate TORC1 through the conserved GTPases, Gtr1 and Gtr2. These form a heterodimeric complex which binds different forms of guanine nucleotides (Valbuena, Guan, &Moreno, 2012). In contrast, mammals have four RAG proteins, where RagA and RagB (Gtr1) share a similar sequence and are functionally redundant, and RagC and RagD (Gtr2) with a high sequence similarity and are functionally equivalent. These pairs form a heterodimer which is important for RAG protein stability as well as mTORC1 activation (Nakashima *et al.*, 1996; Hirose *et al.*, 1998; Jewell *et al.*, 2013).

In *S.cerevisiae,* Vam6 (Vps39 in mammals) acts as a guanine exchange factor in order to activate Gtr1 which then signals to TORC1. When Gtr1 is bound to GTP, and Gtr2 to GDP, this activates signalling to raptor, the subunit of TORC1. The role of Vam6 is not clear but in *S.pombe* a Vam6 mutant had numerous small vesicles which was thought to be due to lack of vacuolar fusion (Valbuena *et al.*, 2012). Vam6 along other Vam proteins are required for the last step of vacuolar assembly where small vacuolar precursors fuse into a large vacuolar compartment which are important for the storage of primary and secondary metabolites (Nakamura *et al.*, 1997; Binda *et al.*, 2009). In mammalian cells however is believed that Ragulator, which is a pentameric complex is responsible for GEF activity and anchoring of the RAG proteins; contains the components p18, p14, mp1, HBXIP and CForf59 (Bar-Peled *et al.*, 2012).

Within the EGO complex, Gtr1 and Gtr2 are known to interact with the proteins Ego1 (also known as Gse2) and Ego3 (also known as Gse1). This complex localises to the endosomal and vacuolar membrane (Walsh, 2010b; Kogan *et al.*, 2010) (Kogan *et al.*, 2010) (see figure 1.12). The EGO complex

controls the exit from rapamycin-induced growth arrest and acts as the counterbalance of the rapamycin-induced, macroautophagy-mediated membrane influx towards the vacuolar membrane. This process is for the resumption of growth following rapamycin treatment (Dubouloz *et al.*, 2005). Currently there is no homologue for Ego1 and Ego3 in mammalian cells. However, Ego3 was shown to closely resemble subunits of the complex containing MP1 and p14 which are also found in the mTOR pathway, detected through X-ray crystallography (Kogan *et al.*, 2010).
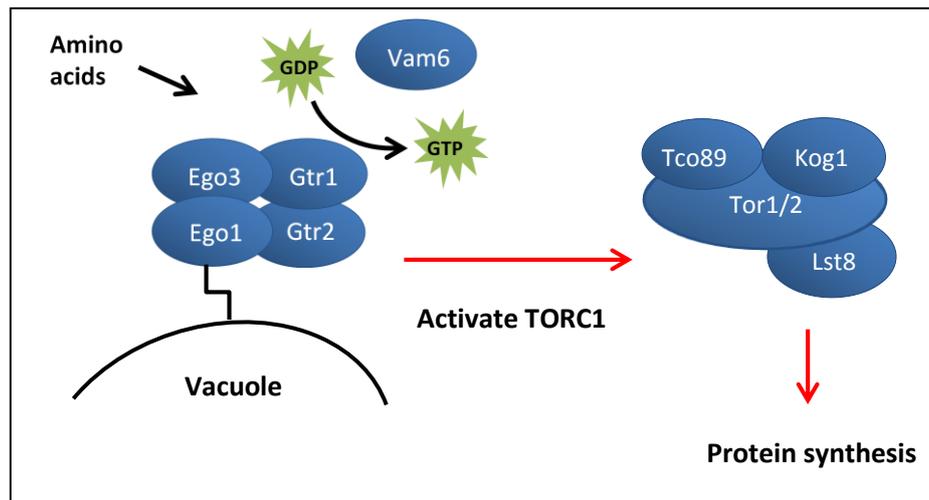
Figure 1.12 The EGO complex in yeast and mammalian systems needed for amino acid sensing in the TORC1 pathway. This complex sits on the vacuole/lysosome. Once Gtr1 is GTP bound, it signals to Kog1 in TORC1 for growth and protein synthesis.

In *S. pombe* cells, Vam6 also co-localises with the GTR proteins at the vacuole membrane where amino acids in the medium stimulates the Gtr1-Gtr2 heterodimer to associate with Mip1 (KOG1 in yeast). When amino acid levels are sufficient and sensed by the EGO complex, TORC1 localises to the vacuole when Gtr1 is GTP bound, however it is unknown how TORC1 moves to the vacuole membrane (Bar-Peled *et al.*, 2012).

The removal of amino acids or even leucine alone from the media inhibits TOR signalling in all eukaryotes. Cells respond to leucine as it is an essential branched chain amino acid and leucine starvation results in the accumulation of uncharged serine and threonine tRNAs as well as leucine tRNAs (Gallinetti *et al.*, 2013) (Kim *et al.*, 2013). In CHO cells, loss of amino acids results in rapid dephosphorylation of S6K (sch9) and 4E-BP1, yet adding back amino acids lacking leucine or arginine, reduces S6K activity by 90% or 70% respectively (Gallinetti *et al.*, 2013). Glutamine plays an important role in growth and metabolism as it is a precursor for nucleotides and other amino acids (Durán *et al.*, 2012). However, glutamine is required for leucine to activate TOR as leucine regulates glutamate

hydrogenase (GDH), the second step involved in glutaminolysis producing α-ketoglutarate (KG) from glutamate. Conversely, if glutaminolysis was inhibited or there is a lack of glutamine, this effects the loading of GTP to RagB preventing localisation of TORC1 to the lysosome and inhibition of protein synthesis (Jewell and Guan, 2013). In yeast, uncharged tRNA are sensed by GCN2 which phosphorylates eIF2α preventing efficient translation initiation. This in turn evokes translation of specific mRNA's such as GCN4, coding a transcription factor activating genes involved in amino acid synthesis (Bushman *et al.*, 1993; Gallinetti *et al.*, 2013; Engelberg *et al.*, 2014).

1.3.4.4 Autophagy is inhibited by TOR

TORC1 signalling stimulates anabolic processes such as translation initiation, but also inhibits catabolic processes such as autophagy. Autophagy is under tight control depending on the cell's energy and nutrition status and is necessary for the degradation and recycling of damaged or redundant proteins and organelles (Scott *et al.*, 2007; Dunlop and Tee, 2013). Protein synthesis is a costly process with an estimated proportion of 30-40% of total ATP (and GTP) energy usage which is why autophagy is important for homeostasis of the cell (Hands *et al.*, 2009)(Mizushima *et al.*, 2008). Autophagy is a pro-survival process as it is induced during ER stress, development, infection and diseases where there is accumulation of protein aggregates. There is cross talk between autophagy and apoptosis as these two pathways are regulated by common components as pro-apoptotic protein Bcl-2 inhibits Beclin 1 disrupting its inhibitory interaction activating autophagy (Pattingre *et al.*, 2005; Mizushima *et al.*, 2008).

There are three types of autophagy based on their mechanism and function, microautophagy where cytosolic components are sequestered directly by the lysosome, macroautophagy where for example organelles are sequestered by vesicle formation known as an autophagasome which can fuse to an endosome or lysosome producing hydrolases breaking down the contents. The last type, chaperone-mediated autophagy (CMA) (only found in mammals) is needed for direct translocation of unfolded proteins which is undertaken in a selective matter (Sakai *et al.*, 1998; Massey *et al.*, 2004; Mizushima *et al.*, 2008).

It is known TOR negatively regulates autophagy as rapamycin treatment activates autophagy even when there are nutrients in the environment. During the initial stages of autophagy, TORC1 directly phosphorylates Atg1 (ULK1/2 in mammals) which when dephosphorylated forms a complex with Atg13; it is not yet fully understood how Atg1 is modulated (Y. Chen & Klionsky, 2011) . However, in mammalian cells and not yeast, the protein focal adhesion kinase family interacting protein of 200 kD (FIP200) was identified in 2008 as an ULK binding protein which forms part of the ATG13-ULK1/2 complex and is needed for stability and phosphorylation of ULK1 (Hara *et al.*, 2008). Autophagy

involves the action of more than 30 ATG proteins which were first identified in yeast, and which are largely conserved amongst eukaryotes (Zustiak *et al.*, 2008; Dunlop and Tee, 2013). The intracellular endomembrane system including the ER, Golgi complex, plasma membrane and the lysosome (or vacuole in yeast) is maintained by dynamic membrane flow between compartments. This is in contrast to autophagy which employs a unique membrane rearrangement. Formation of a vesicle begins with nucleation of a core membrane termed the phagophore, in yeast this occurs at a peri-vacuolar location known as the phagophore assembly site (PAS). However in mammalian cells it is unclear where this site is, but has been observed between the cisternae of the ER (Yorimitsu and Klionsky, 2007). In mammalian cells the two complexes, ULK1-ATG13-FIP200 complex and Beclin-Vps34 complex function to produce the phagophore membrane (initial phase of autophagosome formation) (Mizushima *et al.*, 2003; Dunlop and Tee, 2013).

TOR is not the only pathway controlling autophagy, the PKA pathway is also implicated in its control as elevated levels of PKA activity inhibit autophagy. In *S.cerevisiae* it was discovered that Atg1/Atg13 complex serves as an important point of signal interaction between these pathways as these may respond to distinct cues from different types of starvation (Stephan *et al.*, 2014). These are more examples of how different signalling pathways cross talk for the most effective cellular responses to nutrition and stress in the environment.

1.4 Research objectives of the PhD project

In this project, the focus was on exploiting yeast and CHO systems in the development of screening strategies to select for cell lines with genetic alterations that may improve rP production. A major aim of the project was also to investigate how upstream and downstream regulators of TOR can be manipulated to influence rP production and secretion using yeast and CHO cells. To achieve this, a secreted *Gaussia* luciferase (GLuc) protein was used as a reporter of recombinant protein synthesis in both the yeast and CHO system. The main research objectives were therefore:-

1. **To develop screening strategies for investigate changes to recombinant protein production in yeast**

   A yeast model system for investigating recombinant protein production was developed using a plasmid constructed at The University of Kent containing *Gaussia* luciferase (GLuc). The GLuc assay was utilised to develop a screen where GLuc oxidises a substrate, emitting light which can be measured as an assessment of protein level and activity. Detection and quantification of Gluc expression was also assessed by western blotting. As an alternative we employed a second yeast secretion system that monitors the release of a killer toxin.

2. **Characterisation of TOR and MAPK signalling in relation to the protein cofilin in yeast**

   Cell biological and biochemical methods were used to assess the activity of TOR and MAPK signalling pathways which impact on protein synthesis in response to mutations in the protein cofilin and how these impact upon rP production in yeast. In addition, this objective set out to determine whether mitochondrial function was linked to rP production in yeast.

3. **To analyse changes in TOR signalling for altered protein production through screening methods in yeast**

   Through cell engineering, to investigate how manipulation of upstream and downstream regulators of TOR can either enhance or perturb recombinant protein synthesis. A variety of screening techniques using yeast strains deleted for individual genes within the TOR signalling network were investigated to identify key targets for manipulation of TOR signalling that may affect rP production

4. **To establish whether the effects of manipulation of TOR signalling in yeast can be translated into the CHO cell expression systems**

   TOR is a highly conserved pathway across all eukaryotes. Under this objective it was envisaged to knock down homologous TOR signalling components within the CHO system that were identified as important in yeast using RNAi and determine the influence on recombinant reporter gene expression.

Chapter 2

Materials and Methods

2.1 Yeast Strains, *E. coli* Strains, Mammalian Cell Lines and DNA

2.1.1 Strains

The strains of yeast and their source used throughout this study are described in Table 2.1.1 and 2.1.2 below.

2.1.1.1 Yeast strains

Table 2.1.1 Cofilin strains expressing mutant alleles.

Strain	Genotype	allele	Source/Reference
CGY384	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 COF1::LEU2*	*COF1*	Lappalainen *et al* , 1997
CGY385	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-4::LEU2*	*cof1-4*	Lappalainen *et al* , 1997
CGY386	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-5::LEU2*	*cof1-5*	Lappalainen *et al* , 1997
CGY387	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-6::LEU2*	*cof1-6*	Lappalainen *et al* , 1997
CGY388	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-7::LEU2*	*cof1-7*	Lappalainen *et al* , 1997
CGY389	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof-10::LEU2*	*cof1-10*	Lappalainen *et al* , 1997
CGY390	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof-8::LEU2*	*Cof1-8*	Lappalainen *et al* , 1997
CGY391	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-11::LEU2*	*cof1-11*	Lappalainen *et al* , 1997
CGY392	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-12::LEU2*	*cof1-12*	Lappalainen *et al* , 1997
CGY393	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-13::LEU2*	*cof1-13*	Lappalainen *et al* , 1997
CGY394	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-15::LEU2*	*cof1-15*	Lappalainen *et al* , 1997
CGY395	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-18::LEU2*	*cof1-18*	Lappalainen *et al* , 1997
CGY396	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-19::LEU2*	*cof1-19*	Lappalainen *et al* , 1997
CGY397	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-21::LEU2*	*cof1-21*	Lappalainen *et al* , 1997
CGY398	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-22::LEU2*	*cof1-22*	Lappalainen *et al* , 1997
CGY992	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 COF1::LEU2 Δpdr1::His*	*COF1*	This study
CGY993	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-11::LEU2 Δpdr1::His*	*cof1-11*	This study
CGY994	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-12::LEU2 Δpdr1::His*	*cof1-12*	This study
CGY995	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-15::LEU2*	*cof1-15*	This study

	Δpdr1::His		
CGY996	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-18::LEU2 Δpdr1::His*	*cof1-18*	This study
CGY998	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 COF1::LEU2 ΔSTE12::His*	*COF1*	This study
CGY999	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-4::LEU2 ΔSTE12::His*	*cof1-4*	This study
CGY1000	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-6::LEU2 ΔSTE12::His*	*cof1-6*	This study
CGY1001	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-7::LEU2 ΔSTE12::His*	*cof1-7*	This study
CGY1002	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-11::LEU2 ΔSTE12::His*	*cof1-11*	This study
CGY1003	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-12::LEU2 ΔSTE12::His*	*cof1-12*	This study
CGY1004	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-18::LEU2 ΔSTE12::His*	*cof1-18*	This study
CGY1005	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-19::LEU2 ΔSTE12::His*	*cof1-19*	This study
CGY1006	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-21::LEU2 ΔSTE12::His*	*cof1-21*	This study
CGY1007	*Matα ura3-52 his3Δ200 leu2-3,112 lys2-801 ade2-101 cof1-22::LEU2 ΔSTE12::His*	*cof1-22*	This study

Table 2.1.2 Strains used in this study. *Yeast Mat a Collection was made by the EUROpean *Saccharomyces Cerevisiae* Archive for Functional analysis (EUROSCARF) and purchased from Open Biosystems.

Strain	Genotype	Source/Reference
BY4741	*Mata his3Δ0, leu2Δ0, met15Δ0 ura3Δ0*	CWG collection
ΔAtg1	BY4741 *atg1::KANMX*	Mat a knockout collection*
ΔAtg13	BY4741 *atg13::KANMX*	Mat a knockout collection*
ΔGis1	BY4741 *gis1::KANMX*	Mat a knockout collection*
ΔGln3	BY4741 *gln3::KANMX*	Mat a knockout collection*
ΔGtr1	BY4741 *gtr1::KANMX*	Mat a knockout collection*
ΔGtr2	BY4741 *gtr2::KANMX*	Mat a knockout collection*
ΔHog1	BY4741 *hog1::KANMX*	Mat a knockout collection*
ΔMsn2	BY4741 *msn2::KANMX*	Mat a knockout collection*
ΔMsn4	BY4741 *msn4::KANMX*	Mat a knockout collection*

ΔPkh1	BY4741 *pkh1::KANMX*	Mat a knockout collection*
ΔPkh2	BY4741 *pkh2::KANMX*	Mat a knockout collection*
ΔPDR1	BY4741 *pdr1::KANMX*	Mat a knockout collection*
ΔPDR3	BY4741 *pdr3::KANMX*	Mat a knockout collection*
ΔPDR5	BY4741 *pdr5::KANMX*	Mat a knockout collection*
ΔPDR8	BY4741 *pdr8::KANMX*	Mat a knockout collection*
ΔPDR10	BY4741 *pdr10::KANMX*	Mat a knockout collection*
ΔPDR11	BY4741 *pdr11::KANMX*	Mat a knockout collection*
ΔPDR12	BY4741 *pdr12::KANMX*	Mat a knockout collection*
ΔPDR15	BY4741 *pdr15::KANMX*	Mat a knockout collection*
ΔPDR18	BY4741 *pdr18::KANMX*	Mat a knockout collection*
ΔPkh1	BY4741 *pkh1::KANMX*	Mat a knockout collection*
ΔPkh2	BY4741 *pkh2::KANMX*	Mat a knockout collection*
ΔRps6A	BY4741 *rps6A::KANMX*	Mat a knockout collection*
ΔRps6B	BY4741 *rps6B::KANMX*	Mat a knockout collection*
ΔRtg1	BY4741 *rtg1::KANMX*	Mat a knockout collection*
ΔRtg3	BY4741 *rtg3::KANMX*	Mat a knockout collection*
ΔSfp1	BY4741 *sfp1::KANMX*	Mat a knockout collection*
ΔSit4	BY4741 *sit4::KANMX*	Mat a knockout collection*
SNQ2	BY4741 *snq2::KANMX*	Mat a knockout collection*
ΔTco89	BY4741 *tco89::KANMX*	Mat a knockout collection*
ΔVam6	BY4741 *vam6::KANMX*	Mat a knockout collection*
ΔYOR1	BY4741 *yor1::KANMX*	Mat a knockout collection*
ΔYRR1	BY4741 *yrr1::KANMX*	Mat a knockout collection*
S7	*(dsRNA-)* sensitive strain to K1 toxin	Oliver *et al*, 1977

2.1.1.2. Mammalian Cell Lines

The mammalian CHO cell line engineered to stably express the GLuc reporter gene is described in table 2.2 below.

Table 2.2 CHO cell lines used in this study

Cell Line	Genotype	Source
CHOK1-GLuc	CHO Flip-in GLuc FRT, hygromycin	Invitrogen

2.1.1.3 E.coli strains

The strain of *E.coli* and variations used throughout this study are described in table 2.3 below.

Table 2.3 *E.coli* stains used in this study.

Strain	
DH5α	F- *deoR endA1 recA1 relA1 gyrA96 hsdT17(rk- mk+) phoA supE44 thi-1 Δ(lacZYA-argF)U169 Φ80δlacZΔM15*

2.1.2 DNA

2.1.2.1 Plasmids

The plasmids used throughout this study and their source are described in detail in tables 2.4 and 2.5 below.

2.1.2.1.1 Yeast Plasmids

Table 2.4 Plasmids used in yeast in this study

Name	Description	Reference
pAG415	pAG415*GPD*-ccdB, Gateway expression vector, CEN, *LEU*,	Invitrogen
pCG226	pUG27, *HIS3* LoxP template KO cassette	(Gueldener *et al.*, 2002)
pCG394	*BHUM212 FRE::CYC1::LacZ*, Ste12p activity vector	(Mösch *et al.*, 1999)
pCG483	pRS416- *Sch9*-5HA, *URA*	(Urban *et al.*, 2007)
pCG495	pBevy-U-*gluc1*	Smales Lab
pCG528	VAM6 Donar entry vectors(pDONR221)	PlasmID
pCG529	TCO89 Donar entry vectors(pDONR221	PlasmID
pCG530	GTR1 Donar entry vectors(pDONR221	PlasmID
pCG531	GTR2 Donar entry vectors(pDONR221	PlasmID
pCG532	ATG1 Donar entry vectors(pDONR221	PlasmID
pCG543	pAG415*GPD-ccdB*+VAM6 CEN LEU	This study
pCG545	pAG415*GPD-ccdB*+ TCO89 CEN LEU	This study
pCG546	pAG415*GPD-ccdB*+ GTR1 CEN LEU	This study
pCG547	pAG415*GPD-ccdB*+ GTR2 CEN LEU	This study
pCG548	pAG415*GPD-ccdB*+ ATG1 CEN LEU	This study
pCG556	pVT100U-*KT,* 2μ,URA	(Zhu and Bussey, 1991)
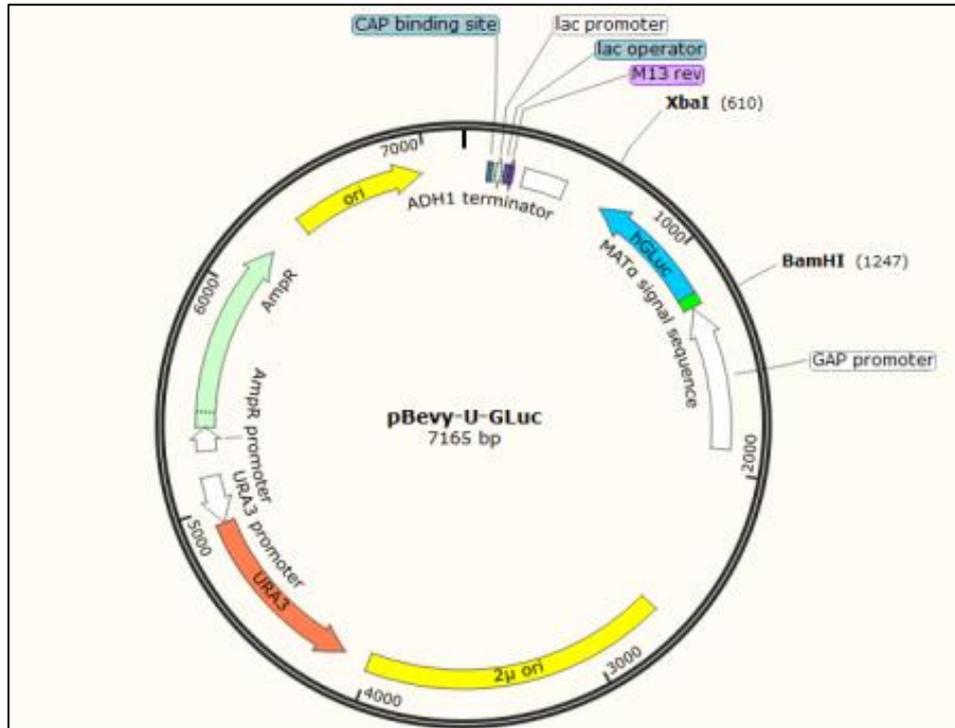
Figure 2.1.1 pCG495 containing the *Gaussia* luciferase gene with the alpha factor signal sequence.
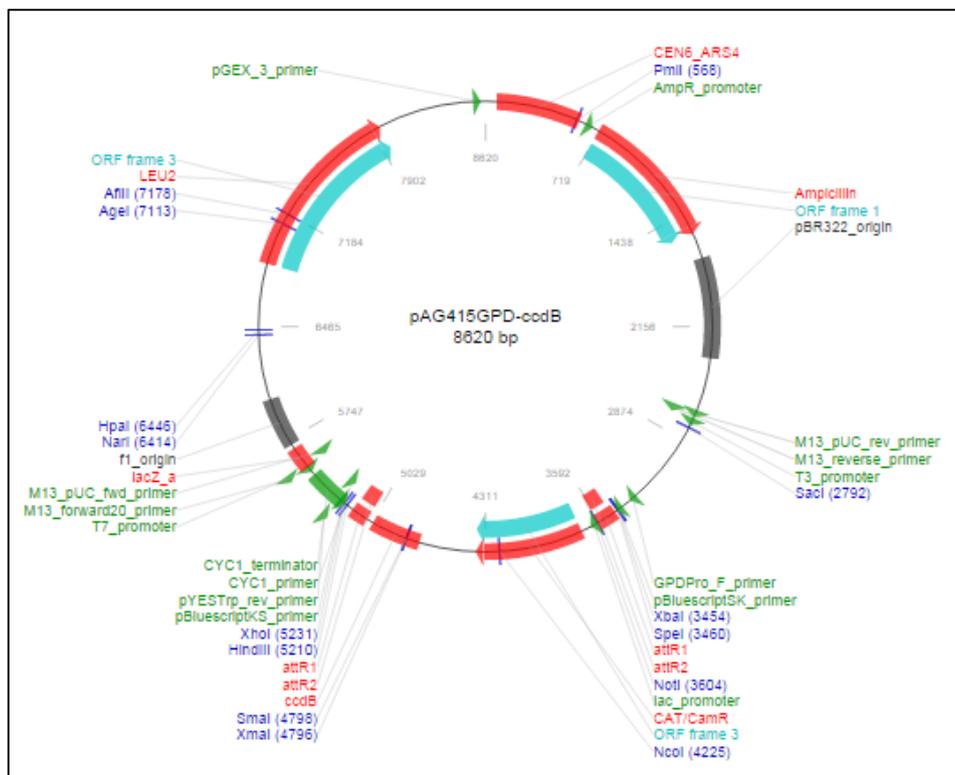
Figure 2.1.2 pAG415 is a destination vector used in the Gateway cloning to create overexpression plasmids used in chapter 5.

2.1.2.1.2 Mammalian Plasmids

Table 2.5 Plasmids used in CHO cell lines.

Name	Description	Reference
pGene clip	pGeneClip Puromycin	Promega
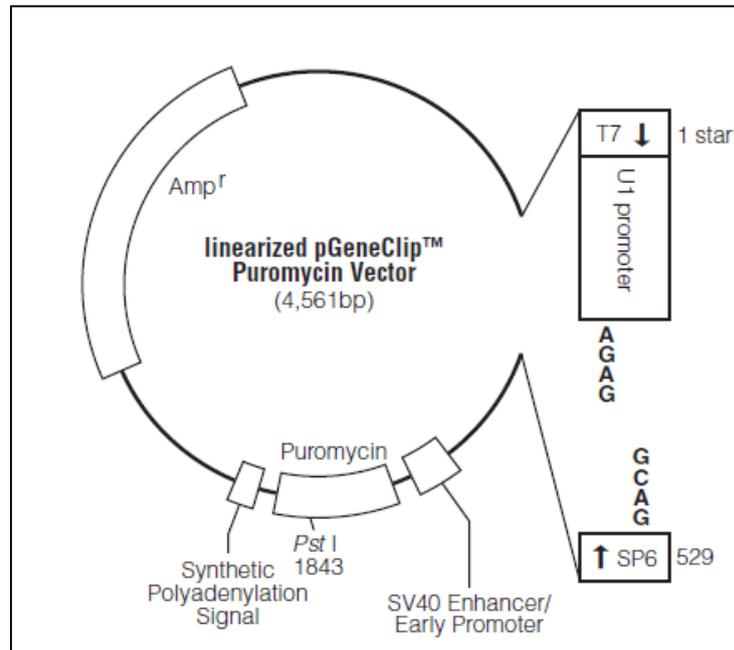
Figure 2.2 pGeneClip^TM Puromycin vector used for knocking down mammalian genes once ligated to hairpin oligonucleotides targeting a specific gene. Oligonucleotides were used from table 2.6.

2.1.2.2 Oligonucleotide Primers

The primers used throughout this study are described in tables 2.6 and 2.7 below. All primers were synthesised by Eurofins MWG Operon.

2.1.2.2.1 Yeast Oligonucleotide Primers

Table 2.6 Yeast Oligonucleotides used in chapter 4 and 5.

Primer	Sequence 5'→3'
PDR1 LoxP Knock out Forward	ATGCGAGGCTTGACACCTAAGAACGGTGTACATATTGAGACGGGCAGTTGAAGCTTCGTACGC
PDR1 LoxP Knock out Reverse	TTAACTATCTGGATAAACGTCGCTCCACAGGATACTGTAGAGGTCGCATAGGCCACTAGTGGATCTG
VAM6 Forward	GAACGCATGTCATCGTATGG
VAM6 Reverse	TATTTCTGCCTTCCCTGGTG
TCO89 Forward	TGCAAGTGCAAAGATTCAGG
TCO89 Reverse	CGTGCATTCTGTGAAGCAGT
GTR1 Forward	TGCCACCATTGATGTAGAGC
GTR1 Reverse	TAACACCTGCACCATCTGGA
GTR2 Forward	TAATGCAACGGACAGGTGAA
GTR2 Reverse	GCTCCGGTATCAGTTTCTGC
ATG1 Forward	CCCCACCGTACAGTCAAACT
ATG1 Reverse	CGGCCAGATTCCAAAATA

2.1.2.2.2. Mammalian Oligonucleotide Primers

Table 2.7 Mammalian primers used for qRT-PCR and oligonucleotides used in cloning to create hairpin inserts (see figure 2.1), for knocking down specific genes in chapter 6 once ligated to the pGeneClip™ Puromycin vector.

Primer	Sequence 5'→3'
Scrambled *ULK1* Hairpin Forward (control)	TCTCGTACCTACGATATTACGGTGCGGCTTCCTGTCACC GCACCGTAATATCGTAGGTACCCT
Scrambled *ULK1* Hairpin Reverse (control)	CTGCAGGTACCTACGATATTACGGTGCGGTGACAGGAA GCCGCACCGTAATATCGTAGGTACC
ULK1 Hairpin Forward	TCTCGCCTGGTTATGGAGTACTGCAACCTTCCTGTCAGT TGCAGTACTCCATAACCAGGCCT
ULK1 Hairpin Reverse	CTGCAGGCCTGGTTATGGAGTACTGCAACTGACAGGAA GGTTGCAGTACTCCATAACCAGGC
Vps39 Hairpin Forward	TCTCGCATGGTGTGTAAGAAGAAGATCTTCCTGTCAATC TTCTTCTTACACACCATGCCT
Vps39 Hairpin Reverse	CTGCAGGCATGGTGTGTAAGAAGAAGATTGACAGGAA GATCTTCTTCTTACACACCATGC
RagC Hairpin Forward	TCTCGGACTTCACATTACTGTTTCTACTTCCTGTCATAGA AACAGTAATGTGAAGCCCT
RagC Hairpin Reverse	CTGCAGGGCTTCACATTACTGTTTCTATGACAGGAAGTA GAAACAGTAATGTGAAGTCC
RagD Hairpin Forward	TCTCGCGGCAAGTCGTCTATTCAGAACTTCCTGTCATTCT GAATAGACGACTTGCCGCCT
RagD Hairpin Reverse	CTGCAGGCGGCAAGTCGTCTATTCAGAATGACAGGAAG TTCTGAATAGACGACTTGCCGC
ULK1 Forward	ACCTATGGAGCATTGGCACC
ULK1 Reverse	CCGTGGGATGGCAGGAACTA
Vps39 Forward	CACTCTCTGGTGCGGAACTG
Vps39 Reverse	TTGATGGTGGGAGTGCCTTC
RagC Forward	GGAGGCTTTAACACGACTTCA
RagC Reverse	TCATTGGCCCTCTGATGAAT
RagD Forward	ACAAAATGTCCCCGAGTGAG
RagD Reverse	GTCAAAGGTGGGGTCAAAAA

2.2 Growth conditions for *Escherichia coli*, *Saccharomyces cerevisiae* and *CHOK1* cells

2.2.1 Growth of *S.cerevisiae*

2.2.1.1 Yeast Extract-Peptone-Dextrose (YPD)
To prepare YP, 2% w/v glucose ; 1% w/v yeast extract (Difco), 2% w/v peptone (Difco), 2% w/v agar (Difco) for solid media.

2.2.1.2 Synthetic minimal without amino acids (synthetic minimal selective medium)
This requires 0.67% w/v Yeast Nitrogen Base without Amino Acids (Sigma), for solid media add 1% Agar w/v (Difco), 2% w/v glucose and Yeast Synthetic Complete Drop-out Media Supplement (Sigma) added to manufacturers' guidelines.

2.2.2 Growth of *E.coli*

2.2.2.1 YT
This requires 1% w/v yeast extract (Difco), 6 g Trypton (Difco), 0.5% w/v NaCl (Fisher) and add 1% w/v agar for solid media. Once cooled to 50°C, antibiotics such as ampicillin (10 µl/ml) can be added.

2.3 Tissue culture techniques

2.3.1 Maintenance of cell lines

2.3.1.1 Routine maintenance of cell lines
Adherent CHO-K1 Flip-in cell lines were maintained in F12 media in flat T flasks (T25, T75 and T175; Starstedt). The F12 HAM (Gibco) also contained 6 mM L-glutamine, 10 ug/ml Hygromycin and 10% Heat inactivated foetal bovine serum (FBS) (Lonza). Cells were grown at 37°C in 5% CO_2 atmosphere.

All cells were passaged at 70% confluence. The cells were washed with 1xPBS (Oxoid) and detached using 0.25% trypsin which was left at 37°C for up to 5 minutes. To inactivate the trypsin once the cells have detached, serum containing medium was added. Adherent cells were cultured in vented flasks (Sarstedt) and welled plates (CELLSTAR, Grenier Bio-one).

2.3.1.2 Cryopreservation and recovery of cells

Cells in exponential phase of growth were washed in PBS and treated with 0.25% trypsin. These were then centrifuged at 1000 rpm for 5 minutes and resuspended in media containing 10% dimethyl sulfomine (DMSO, Sigma-Aldrich). Cryotubes were frozen at -80°C overnight then transferred to liquid nitrogen.

To revive cells, the cells were warmed to 37°C to thaw and then 9 ml of media (at 37°C) added. Next the cells were centrifuged again at 1000 rpm for 5 minutes, and the pellet resuspended in 1 ml of media. This was transferred to a T25 flask containing 9 ml of media.

2.3.2 Transient expression of plasmids

In a 6 well plate, cells were seeded at $5x10^5$ viable cells per ml of growth media. 24 hours later cells were washed and new media added. For each transfection, 1 µg of DNA was mixed with 250 µl of Optimum (Invitrogen), and in another tube 12 µl of lipofectamine 2000 (Invitrogen) was mixed with 250 µl Optimum. These were left for 5 minutes at RT then mixed and incubated for 20 minutes at RT. 500 µl of this mixture was added to the well and incubated at 37°C for 4 hours, after which the media was changed.

2.4 Yeast Genetic techniques

2.4.1 Yeast transformations by heat shock

With exogenous DNA, yeast cells were grown overnight and 1 ml of the culture was pelleted in a table-top centrifuge for 4 minutes at 3000 rpm. After the supernatant was removed, the cells were resuspended in 1 ml of TE buffer (pH 9), and then pelleted and the supernatant removed again. The cells were then re-suspended in 0.1 ml of 0.1 M lithium acetate (LiOAc) in TE. Next, 15 µl of carrier DNA was added (10 mg/ml single stranded DNA) followed by 1-2 µl of the transforming DNA and mixed gently. 700 µl of 40% PEG4000 (in 0.1 M LiOAc in TE) was added and the sample incubated with rotation for at least an hour on a roller. Next, the samples were incubated for 15 minutes at 42°C. After this time, the samples were centrifuged and the pellet resuspended in 200 µl of sterile H_2O and plated on selective agar. These plates were left to grow at 30°C.

2.4.2 Generation of LoxP marker cassettes for yeast gene knock outs

A gene disruption cassette can be used to delete a specific gene in budding yeast. This gene disruption cassette contains a selection marker gene (for example *HIS3*) which is flanked by two *loxP* sequences which are adjacent to the 45 bp of sequence flanking the gene you wish to delete. PCR can be used to amplify the cassette by using oligonucleotides that incorporate 19 or 22 3' nucleotides complementary to the *loxP* sequence flanking the marker and to the sequences in the template flanking the disruption cassette and then 45 5' nucleotides that anneal with sites upstream and downstream of the target sequence. Once this disruption cassette is ready, it is purified using the phenol/chloroform extraction method in section 2.5.4. This cassette was then transformed into the cells and checked using PCR for the correct integration of the disruption cassette. For this, primers are used that are complementary to the sequences in the cassette as well as one complementary to the sequence within the target gene's sequence. This method can be seen in figure 2.2 below.
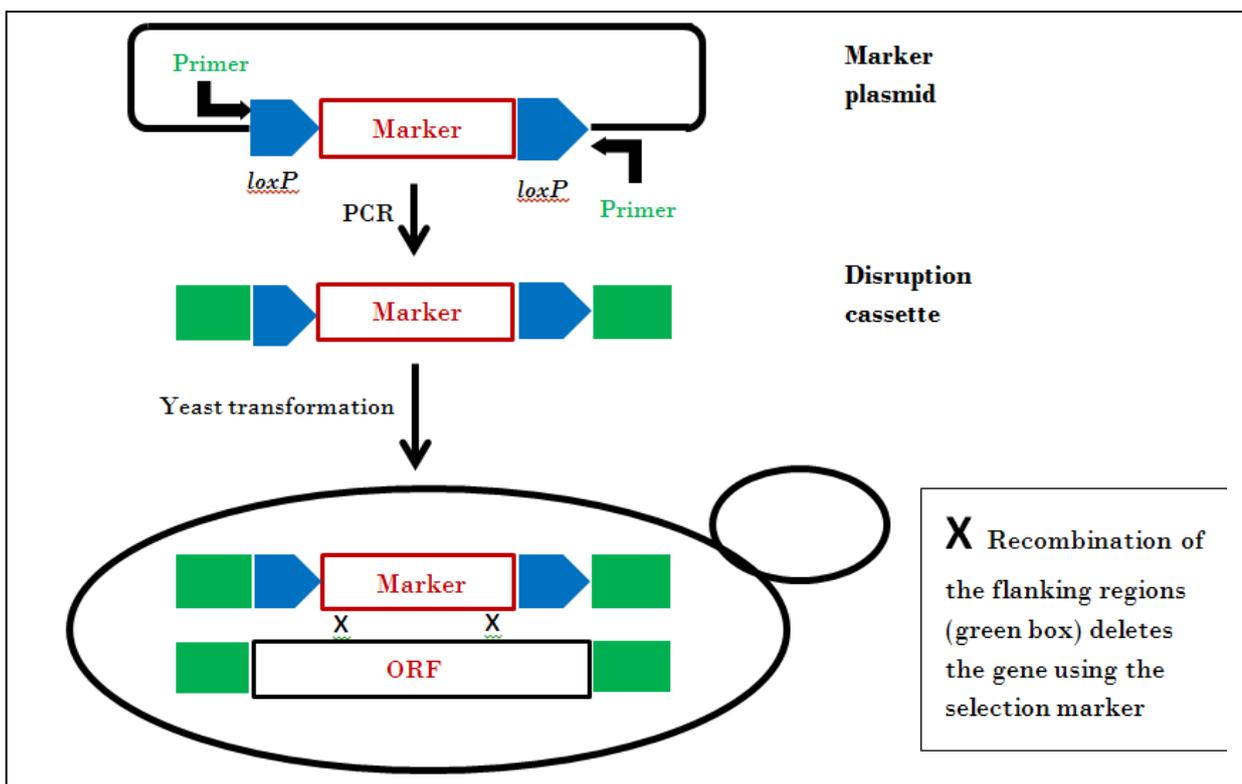
Figure 2.3 Schematic using the LoxP marker cassette for disrupting a target gene.

2.4.3 Generation of petite ρ0 (rho) yeast strains

This method was undertaken to create yeast without mitochondrial DNA. The cells were grown in liquid YPD medium overnight containing sterile ethidium bromide (EtBr) at 25 μg/ml. These cells were taken from cells grown on YPD agar. The following day, a fresh culture was inoculated using the overnight cells in YPD containing EtBr at the same concentration as before. These cells were left to grow overnight until they have reached saturation. 10-20 μl was streaked out on YPD agar.

2.5 Molecular biology techniques

2.5.1 Miniprep purification of DNA

This process used 1 ml of overnight cultures of bacterial strains containing the plasmid DNA required. The purification of plasmid DNA was achieved using the Quiagen Qiaprep® Miniprep kit where the cell lysate was placed in the silica spin column following the instructions provided by the manufacturer. A number of buffers with high salt concentrations were used so the DNA absorbs into the silica matrix but remaining contaminants in the lysate were passed through the column and removed. The DNA that was bound was washed with an ethanol mixture followed by elution of the DNA through the column (refer to Qiagen's Qiaprep® Miniprep kit handbook for more information.)

2.5.2 Polymerase Chain Reaction (PCR)

PCR reactions are used to amplify specific DNA sequences such as the HIS deletion cassette. The essential components for this reaction include a DNA polymerase enzyme (such as Taq from Roche), nucleotides, a DNA template and oligonucleotides (these are all described in Tables 2.8). Using a Thermocycler, the reactions can take place at specific temperatures for exact periods of time. This reaction amplifies DNA by using DNA polymerase enzyme which will generate single stranded DNA from the 5' to 3' ends where the primers indicate the specific starting point. The changes in temperature lead to elongation, annealing and denaturation of the DNA. This produces templates which will then generate more fragments correlating to the primer nucleotides that were on the original DNA template used.

Table 2.8 The reaction mixture contained the following reagents to make 25 ul reaction tube:

Reagents	Volume
10x PCR buffer (Invitrogen)	2.5 µl
10 mM primer	0.5 µl
10 mM dNTPs (Invitrogen)	0.5 µl
~1 µg template DNA	variable volume
Taq DNA polymerase (5units/µl) (Invitrogen)	0.125 µl
nuclease free water	Make up to 25 µl

An example of the programme used for the thermocycler is shown in Table 2.9 below:

Table 2.9 Temperatures and timing of each step in PCR.

95°C for 3 minutes	Initial Denaturation step of template
95°C for 30 seconds	Short Denaturation ⎤ Repeat cycle
54°C (varies depending on primers) for 1 min	Annealing ⎦ 30x
72°C for 2 minutes	Elongation
72°C for 10 min	Final Elongation

2.5.3 Agarose Gel electrophoresis Analysis of DNA

Agarose gel electrophoresis was used to separate and analyse various DNA fragments and plasmids by size. The gel was made up of 1% (w/v) agarose (Melford) in TAE buffer. Reagents for TAE buffer include 40 mM tris, 1 mM EDTA and 20 mM Acetic acid, pH 8.5).

The agarose-TAE mixture was placed in the microwave at full power for 1-2 minutes to dissolve the agarose. Once this has cooled, 0.5 µl of a 10 mg/ml ethidium bromide (EtBr) solution was added to 35 ml of agarose solution and mixed. This mixture was then poured into a casting apparatus to form a gel with the comb. After the gel was set it was placed in an electrophoresis tank. The gel was submerged

in TAE making sure it covered the electrodes on each end of the tank; this was then ready for the samples to be loaded. Each sample was mixed with 5-7 μl of the concentrated DNA with 0.5-1 μl of 6x sample loading dye (Promega, G190A) and then pipetted into a well. A 1 kb DNA ladder (Promega, G571A) as a DNA standard was run alongside the samples. The samples were usually run for 20 minutes at 120 volts or until the dye had migrated to the bottom of the gel. Once the gel was run, the fluorescence of the samples and ladder were observed on a short wave transilluminator (312 nm) and an image generated by a digital camera.

2.5.4 Phenol chloroform purification of DNA

A phenol/chloroform extraction method was used to purify the PCR product of the HIS knock out cassette. This method separates materials (i.e. proteins and DNA from generation of DNA cassettes through PCR) in an organic (i.e. phenol chloroform) and inorganic phase. First, equal volumes of buffer-saturated phenol:chloroform (1:1) were added to the same volume of DNA and vortexed, followed by 3 minutes in a table-top centrifuge at maximum speed. Next, the aqueous layer was carefully removed and transferred to a new microcentifuge tube (spin again if not all the aqueous layer is removed). An ethanol precipitation method was used to concentrate the DNA further by adding 3 volumes of ethanol (100%) to the DNA sample which was then incubated at -20°C overnight for full precipitation. To extract the precipitated DNA the tube was spun for 10 minutes in a table-top centrifuge at full speed. The supernatant was discarded, and the DNA pellet formed was washed with 70% ethanol solution and spun down again (10 minutes at full speed). After the pellet was left to air dry, it was re-suspended in 20 μl of TE buffer at pH8.

2.5.5 *E.coli* competent cells

DH5α cells were grown overnight in 10 ml broth in a shake flask for aeration. The next day 8 μl of the culture was used to inoculate 28 ml of broth and growth with aeration at 37°C. After approximately 4.5 hours, the culture reaches an OD_{600} of approximately 0.5. Five minutes before the culture reaches this cell density, 3.75 ml of sterile warm 100% glycerol was added slowly. The cells were then chilled on ice for 10 minutes and then pelleted at 4°C for 10 minutes at 4000 rpm. Next, the supernatant was poured off and the cells resuspended in an equal volume of ice-cold 0.1 M $MgCl_2$ plus glycerol (0.1 M $MgCl_2$ and 15% glycerol). The cells were then pelleted at 4°C for 8 minutes at 3800 rpm and the supernatant removed. The cells were then resuspended in 6.25 ml of ice-cold T-salts (0.075 M $CaCl_2$, 0.006 M $MgCl_2$ and 15% glycerol) and incubated on ice for 20 minutes with occasional mixing. This

then centrifuged at 4°C for 6 minutes at 3600 rpm and resuspended in 1.25 ml of T-salts. This can be stored at -80°C. This must be left to freeze overnight before use.

2.5.6 *E.coli* transformation

50 µl of competent cells were first thawed on ice before adding 1 µg of DNA. This mixture was incubated for 30 minutes on ice followed by heat shock for 30 seconds at 42°C. 450 µl of YT media was added and incubated at 37°C with shaking. This was then plated onto YT agar plates. 10 µg/ml of Ampicilian was used for plasmid selection.

2.6 Protein analysis techniques

2.6.1 Polyacrylamide Gel Electrophoresis (PAGE)

2.6.1.1 Preparation of protein samples

2.6.1.1.1 Yeast whole cell extract method

Cells were gown overnight in media. The next day samples were diluted to the same cell density. 200 µl of the culture were pelleted at 4000 rpm for 3 minutes using a table-top centrifuge. After the supernatant was removed, about 100 µl of glass beads were added and mixed. 25 µl of 2x sample buffer (2.5 ml of 0.5 M Tris pH6.8, 2 ml 10% SDS, 2 ml ddH$_2$O, 1 ml β-mercaptoethanol, 0.5 ml Bromophenol blue) were then added and vortexed for about 2 minutes. Next, the samples were boiled for 3 minutes after which 100 µl of 2x sample buffer was added once again and vortex. The samples can then be used immediately or stored at -20°C.

2.6.1.1.2 Urea-TCA protein extraction for Sch9 analysis

Due to the large size of the protein Sch9 (~100 kDa) and the presence of multiple phosphorylation sites, a separate protocol was used for the protein extraction using NTCB (2-nitro-5-thiocyanatobenzoic acid) which selectively cyanylates cysteine residues allowing analysis of smaller fragments. This method was taken from Soulard et al., 2007. The method was used to extract protein from cells in log phase and post-diauxic shift. Cells were grown in overnight culture and the following morning 1 ml of the culture was re-inoculated into 10 ml of media; these cells grew into log phase after 3-5 hours. The remaining overnight culture was diluted to an OD$_{600}$ of about 0.3 and left to grow for 24 hours before protein extraction.

For the protein extraction, 1 ml of the cells was used at an OD_{600} of 0.8-1 for each sample. Some samples were left to grow at 30°C in 200 ng/ml of rapamycin for 30 minutes. After this time, 66 µl of TCA (100%) was added and then put on ice for at least 10 minutes. The samples were then spun at 13000 rpm and the supernatant discarded. The cells were washed in 200 ul of cold acetone, sonicated for 30 seconds and spun quickly in a tabletop centrifuge at high speed. After the supernatant was discarded, the cells were washed in acetone again, spun down, and dried in a speed vac for 5 minutes at about 60°C. The pellet was re-suspended using 10 µl of the urea buffer (50 mM Tris-HCL pH7.5, 5 mM EDTA, 6 M urea, 1% (w/v) SDS, 1 mM PMSF, 10 mM Phosphatase inhibitors (Sodium flouride, β-Glycerophosphate disodium salt hydrate, Sodium pyrophosphate and Sodium orthovanadate)) and glass beads added up to the liquid surface. However only add PMSF and PPi just before use. Cells were lysed in the bead beater for three rounds of 10 seconds in between 60 second pauses. Next, to remove the bubbles, the samples were spun in the table-top centrifuge for 5 minutes followed by 10 minute incubation at 65°C. 3 µl of 0.5 M CHES (N-cyclohexyl-2-aminoethansufonic acid) and 2 µl of 7.5 mM NTCB was added and each sample was vortexed. After the samples were left at room temperature overnight, 15 µl of 2x Laemmli Buffer (100 mM Tris-HCL pH 6.8, 4% SDS, 20% glycerol, 0.02% Bromophenol Blue, 200 mM DTT) was added and the samples boiled for 10 minutes at 65°C. These samples were then run on a 7.5% acrylamide gel.

2.6.1.1.3 Yeast whole cell quantitative method

This method was taken from Von der Haar, 2007. Cells were grown overnight in minimal media and inoculated the following day in YPD and grown to a cell count of 1×10^7. If supernatant was needed, 500 µl of the sample was added to 500 µl of sample buffer and vortexed. Pellet samples were spun down for 2 minutes at high speed and harvested followed by re-suspension in 200 µl of Lysis Buffer (0.1 NaOH, 0.05 M EDTA, 2% SDA, 2% β-mercaptoethanol). The samples were then incubated for 10 minutes at 90°C with 50 µl of loading buffer (0.25 M Tris-HCL pH 6.8, 50% glycerol, 0.05% Bromophenol Blue). Next the samples were centrifuged to clear the lysate and the extract loaded onto the gel or frozen at -20°C for future use.

2.6.1.1.4 Mammalian cell lysis

Cells are grown in 24 well plates (Greiner) to >70% confluence then the media removed. If the supernatant was needed, 1 ml was retained and 200 µl of lysis buffer added and placed immediately on ice. The cells were washed with PBS to wash off any excess media. 400 µl of 0.25% trypsin solution was then added and left at 37°C for up to 5 minutes until all the cells became dislodged from the surface. Next, 800 µl of media was added. 500 µl was put in a tube and spun down for 2 minutes at high speed and the supernatant removed. The pellet was resuspended in 200 µl of lysis buffer and left on ice for 5 minutes before stored at -80°C.

500ml of Lysis buffer contained 200 mM HEPES pH 7.2, 100 mM NaCl, 1% TX 100 and 10 mM Na β-glycerophoshate. Phosphatase and protease inhibitors were added to an aliquot of lysis buffer when needed. For 10 ml Lysis Buffer with inhibitors, 50 mM NaF, 1 mMNa-Vanidate (New England Biolabs) and 1 EDTA-free protease inhibitor tablet (Roche) were added.

2.6.1.1.5 Determination of protein concentration using the Bradford assay

The concentration of proteins in soluble extracts was determined using the commercial Bradford reagent (Biorad). 2 μl of protein sample was added to 48 μl of dH$_2$O and 1 ml of Bradford reagent added, vortexed, and left for 10 minutes. The sample was then read at A$_{595}$ nm and the concentrations of samples determined with comparisons to known concentrations in a standard curve generated using known amounts of bovine serum albumin (BSA).

2.6.1.2 Preparation of polyacrylamide gel

The polyacrylamide gels used had a 5% acrylamide stacking gel on top (see makeup below) and a resolving gel on the bottom that can have varied concentrations between 7.5%-15% acrylamide. The resolving gel mixture was poured into the BioRad cast system and covered with isopropanol for the gel to polymerise correctly. When the gel was polymerised and the isopropanol poured out, the stacking gel was poured on top of the resolving cell and a comb placed on top for the sample wells to form as the gel sets. The reagents and solutions required are described in the table 2.10 below.

Table 2.10 Reagents of acrylamide gels. Columns show varied percentage of acrylamide used, gel size 9x9cm.

Solution	7.5%	10%	12.5%	15%	5% (Stacking only)
Acrylamide 40%	1.5	2.0	2.5	3.0	0.375
1.5 M Tris (pH 8.8), containing 0.4% SDS	2.0	2.0	2.0	2.0	
0.5 M Tris (pH 6.8), containing 0.4% SDS					0.75
dH$_2$O	4.5	4.0	3.5	3.0	1.875
TEMED (μl)	5	5	5	5	3.5
10% APS (μl)	35	35	35	35	35

2.6.1.3 Polyacrylamide gel electrophoresis protocol

When the gel had polymerised, it was ready for electrophoresis. The gels were placed in the electrophoresis tank and immersed in Tris/Glycine/SDS buffer (TGS). After 5 µl of the protein ladder and 10-20 µl of samples where loaded the gels were run with a voltage at 90 V until the tracking dye had run through the stacking gel and entered the resolving gel, at this point the voltage was increased to 120 V. The samples took between 2 and 2.5 hours to run. The gels were stained with Coomassie Blue to visualise the proteins present on the gel or used for the next step of a western blot as described below.

2.6.2 Western Blot

The western blot procedure consisted of a semi-dry transfer onto a PVDF membrane and then the immunoblotting procedure for binding antibodies to the protein on the membrane which can then be detected using Chemiluminescence (ECL).

2.6.2.1 Semi-dry protein transfer

Initially the PVDF membrane was soaked thoroughly in methanol and then left to soak in transfer buffer (0.0029% w/v Glycine, 0.00004% w/v SDS, 0.0058% w/v Tris Base, 20% v/v methanol) for 10-15 minutes. On the bottom electrode of the Bio-Rad semi-transfer apparatus, a piece of thick blotting paper soaked in transfer buffer was placed first, taking care that any air bubbles were rolled out. Next the PVDF was placed neatly on top of the blotting paper, and then the gel on top of that. The next layer placed was again a piece of thick blotting paper soaked in transfer buffer. Once this was done then the top electrode can be placed on the apparatus and connected to the power supply. This was run at 25 V for 15-30 minutes per membrane. Once transferred the membrane was ready for the immunoblotting procedure.

2.6.2.2 Immunoblotting

Once the transfer was complete, the membrane was placed in blocking solution (5% w/v dried skimmed Milk in PBS/T containing Phosphate Buffered Saline with 0.2% Tween 20) and shaken for 45 minutes at room temperature. Next the membrane was washed in PBS/T and placed in a 50 ml falcon tube with 3-20 ml blocking solution containing the desired concentration of primary antibody and left on a roller for an hour at room temperature (it can be left at 4°C overnight on the roller). The membrane was then rinsed twice in PBS/T, then left to wash in PBS/T for 15 minutes with gentle shaking, followed by two 5 minute washes. Next the membrane was left in blocking solution containing the secondary antibody in a falcon tube for 20-30 minutes. The secondary antibody was

conjugated to Horse-Radish Peroxidase. The membrane was then washed in PBS/T as before to remove unbound primary antibody. This was followed by an extra wash for 5 minutes before leaving in PBS (Phosphate Buffered Saline) and ready for detection (see section 2.5.2.3).

2.6.2.3 Western blot detection – ECL method

In the dark room, proteins were detected using the Chemiluminescent (ECL) method where the membrane was left in the developing agents (mixed at a 1:1 ratio) for 1 minute. Solution 1 contains 1 ml luminol 250mM (3-aminophthalhydrazide for FLUKA No.09253), 0.44ml *p*-coumaric acid 90mM (Sigma), 10ml 1M Tris-HCL (pH8.5) with water to 100ml). Solution II contains 64µl 30% hydrogen peroxide, 10ml 1M Tris-HCL (pH8.5) and H_2O made up to 100ml.

The membrane was then dabbed on tissue to remove excess liquid and placed in a plastic wrap that is in a film development cassette. The ECL film was placed on top of Saran wrap containing the membrane and the cassette shut for the required time to be exposed. After the required time, the film was transferred to the development machine (XOgraph Compact X4).

2.6.2.4 Antibodies used for protein detection

The primary antibodies used for the detection of actin were raised in mouse and diluted 1:5000. This antibody was a gift from Prof. John Cooper (Department of Cell Biology & Physiology Washington University in St Louis). The GLuc antibodies (New England Biolabs) used were raised in rabbit and diluted 1:3000. Antibodies were diluted 1:5000 to detect PGK and raised in rabbit; this was purchased from York Biosciences. Anti-HA was developed in rabbit and diluted to 1:1000 for detecting Sch9 which was tagged (Sigma-Aldrich). The p38/Hog1 MAPK antibody was a gift from Prof. Nic Jones (Paterson Institute for Cancer Research, Manchester) and diluted 1:1000. In detecting phospho-p38 MAPK, an antibody was purchased from Cell Signalling Technology which was raised in rabbit and diluted 1:1000.

2.6.3 Gaussia luciferase assay

This reporter assay uses the protein *Gaussia* luciferase (GLuc) from the organism *Gaussia principe.* The luciferase protein catalyses the oxidation of the substrate coelenterazine in a reaction emitting light. This technique used the commercially available kit from Invitrogen. Overnight cultures were grown in the appropriate minimal media and reinoculated to grow them into log phase. After 3-4 hours, $3x10^5$ cells were harvested and spun down at 3000 rpm for 4 minutes. 25 µl of supernatant was then placed in a 94 well plate. The BioLux Gluc substrate was mixed with the buffer (100X) diluting it by 100-fold and left on ice. 25 µl of this was delivered to each well using a multichannel pipette. Measurements

were promptly taken on the BMG Optima Fluostar mulitplate reader at 475 nm. More details on the settings of the plate reader are shown below in Table 2.11.

Table 2.11 Optima settings used for Optima Fluostar mutiplate reader during the GLuc assay

Optima settings
No. of multichromatics =1
Emmission filter = Lens
Gain = 3600
Optic = top optic
Positioning delay = 0.2
No. of kinetic windows = 1
No. of cycles = 1
Measurement start time = 0.0 sec
Time to normalise the result = 0.00 sec
Pass before cycle = 0.00 sec
Shaking = orbital 500 rpm
Additional shaking = before each cycle
Shaking time = 2.0 sec

2.7 RNA analysis technique

2.7.1 Total RNA extraction

2.7.1.1 Total RNA extraction from CHO

Cells grown in 24 well plates had the media removed and were washed with 1 ml PBS, then 400 μl of trypsin(Invitrogen) was added and incubated at 37°C for up to 5 minutes until the cells had been dislodged. 800 μl of media were then added, the cells were spun down and the supernatant removed. The pellet was resuspended in RLT (Qiagen) (600 μl for $5x10^6$ to $5x10^7$ cells). The RLT lysate was then homogenised with QIAshredders (Qiagen). RNA was extracted according to the instructions from RNeasy mini kit and RNA levels measured using the Nanodrop spectrophotometer (Labtech). These RNA extracts were stored at -80°C.

2.7.1.2 Total RNA extraction from yeast

Cells were grown overnight in minimal media then inoculated into YPD and grown to the cell count $1x10^7$/ml. Cells were treated according to the RNeasy mini kit (Qiagen) using the mechanical disruption technique where acid-washed glass beads are used to break open the cells.

2.7.1.3 DNase treatment of RNA samples

RNA samples were treated with the RQ1 RNase-free DNase kit from Promega to degrade any DNA contamination. See protocol for more details.

2.7.2 qRT-PCR

The method qRT-PCR was used to determine relative amounts of target mRNAs. β-actin was used as the house keeping mRNA. qRT-PCR was carried out using the Quantifast SYBR Green PCR kit from Qiagen. The dye SYBR Green 1 was used binding to double stranded DNA so fluorescence is detected of amplification products. Below in table 2.12 is the reaction mixture needed per reaction. 96 well plates were used with an adhesive seal and run using the MiniOpticon Sytem (BioRad).

Table 2.12 Reagents needed for qRT-PCR

Reagents per well	Volume	Final concentration
2X Quantifast SYBR Green PCR Master Mix	12.5µl	1x
Forward primer	Variable	1 µM
Reverse primer	Variable	1 µM
Template RNA	Variable	≤100 ng/reaction
RNase-free water	Variable	
Total reaction volume	25 µl	

The resulting data was analysed using Opticon Monitor software. Melting curve analysis can be undertaken to analyse product homogeneity after a run. Normalised data was exported to Microsoft Excel for plotting graphs.

Table 2.13 Temperature and time of each step in qRT-PCR.

Step	Temp/time
Initial denaturation	98°C for 30 sec
Denaturation	98°C for 10 sec
Annealing	Variable for 30 sec
Extension	72°C 30 sec/500 bp
Extension final	72°C 5 -10 min

Repeat cycle 35x (Denaturation and Annealing)

2.8 Phenotypic analysis techniques

2.8.1 Multi-well plate growth assay

To study the growth of cells, spectrophotometric growth readings were taken using the BMG LABTECH SPECTROstar Nano plate reader where optimal density (OD) measurements were taken through a 595 nm(~600) visible light path. 5 ml culture was grown overnight and diluted with the starting OD_{600} of 0.1-0.3 in bio-one well plates (Greiner). The cell density was determined by the Eppendorf BioPhotometer plus. The basic settings are described in Table 2.14 below:

Table 2.14 Standard settings for multi-well plate growth assay of yeast.

Typical protocol settings
Flashes per well: 3
Cycle time (sec): 1800
Excitation: 600
Shaking frequency (rpm): 400
Shaking mode: double orbital
Additional shaking time: 30 s before each cycle
Positioning delay: 0.5
Target Temperature (°C): 30

Data was exported from MARS data analysis software (BMG labtech) to Microsoft Excel and used to generate the growth curves from the readings. Data was corrected with the bank before logging the readings and calculating the maximal growth rate over a 3 hour period. The OD reading at the earliest point within a 3 hour time frame was subtracted by the final point and divided by 3. The doubling time was calculated by dividing 0.693 by the growth rate.

2.8.2 β-galactosidase activity assay

This method uses a *LacZ* expression vector which is a reporter construct used in this study to measure Ste12 activity. For this, cells were grown overnight in selective media. These cultures were then inoculated in YPD cultures at an OD_{600} of 0.3 and incubated at 30°C overnight. 200 µl of these cells at an OD_{600} of 5 were added to Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCL, 1 mM Mg_2SO_4, 50 mM β-mercaptoethanol) to make up the volume to 600 µl. This mixture was incubated at 30°C for 30 minutes. After this time, 150 µl of 4 µg/ml ONPG (2-nitrophenyl β-D-galactophyranoside) (Sigma) was added and gently shaken. The reaction was then stopped by adding 400 µl of 1.5 M Na_2CO_3 (depending on the amount of protein used will depend on how long to wait before stopping the reaction). Samples were then centrifuged for 30 seconds at 13,000 rpm to remove cell debris before the absorbance was read through a spectrophotometer at 420 nm and 550 nm.

The following equation was used to calculate the miller units of the protein in question:

$$\text{Miller unit} = 1000 \times \left(\frac{\text{Absorbance at 420} - (1.75 \times \text{Absorbance at 550})}{t \times v \times \text{Absorbance at 600}} \right)$$

t = the time left for the reaction to occur before stopped by Na_2CO_3

v = the total volume of cells and Z buffer

2.8.3 Spotting assay

Cells were grown overnight in the appropriate media and diluted to an OD_{600} of 0.075 in 550 µl sterile water in a sterile autoclaved steel multi-well plate. Using flat-bottomed steel pins, cells were transferred to dried agar plates. This was left to grow at 30°C for 2-3 days.

2.8.4 Killer Toxin assay

This method uses a plasmid containing K1, a killer toxin naturally found in certain yeast isolates. Strains containing this plasmid are grown on a lawn of yeast cells sensitive to the toxin on agar plates forming a kill zone. These agar plates contain methylene blue, a dye which stains dead cells. This kill zone is then measured using the image processing programme Image J.

Initially to prepare these plates the bottom layer of agar was set, followed by a top layer (lower pH to 4.8) containing methylene blue and the sensitive strain S7 added after autoclaving. Table 2.15 below describes the makeup for each agar layer. The bottom layer can be made at an earlier time. All yeast strains are grown overnight in minimal media. The following morning, the sensitive strain was diluted

back to an OD_{600} 0.05 and left to grow into early log phase with a cell count of about $2x10^6$. The killer strains are then diluted to an OD_{600} of 0.2 and grown to a cell count of $1x10^7$. While the strains are growing, the top agar was made and left to cool to 45°C. Once the sensitive strain had grown to the appropriate cell count, 500 μl was added per 10 ml of top layer media in a falcon tube. The plates were warmed containing the bottom layer before pouring over the top layer taking care to spread it evenly before it sets. Once the killer strains have grown to the correct cell count, cells were diluted in 2 ml with the cell count adjusted to $4x10^6$ and further dilutions as required. Cells were spun down at 4000 rpm for 4 minutes. The supernatant was removed and pellet resuspended in 60 μl sterile water. 10 μl of the cell suspension was then spotted onto small filter disks (0.5 cm diameter) on the top layer. The plates were then allowed to dry and incubated at 30°C. Plates were scanned after 2-3 days growth.

Table 2.15 Reagents included in Top agar and Bottom agar used in the killer toxin assay.

Reagents	Top agar (100 ml)	Bottom agar (100 ml)
Yeast extract	0.5 g	0.5 g
Bactopeptone	0.5 g	0.5 g
Agar	1.5 g	0.5 g
Glucose	5 ml	5 ml
Methylene blue	1ml	

2.9 Cloning

2.9.1 Gateway Entry cloning

Gateway Technology by Invitogen was used to perform an LR recombination reaction between an attL-containing entry clone at each end of the gene (see Figure 2.3). The attL sites are complementary to the sticky ends of the destination vector, attR. The attL sites on the entry clone are cut by the gateway recombination enzymes, leaving sticky ends which recombine with the attR sites of the destination vector creating the attB containing expression clone and attP by product. Reaction solutions are described in table 2.16 below:

Table 2.16 Reaction solution in Gateway cloning.

Reagents	Volume
Entry clone (100-300 ng/reaction)	1-10 µl
Destination vector (300 ng/reaction)	2 µl
Reaction buffer	2 µl
LR clonase II	4 µl
TE buffer pH8	Make up to 16 µl

The mixture was incubated at room temperature for 1 hour followed by 1 µl of proteinase K added and incubated for 10 minutes at 37°C. This stops the reaction by destroying the clonase enzyme. This was then transformed into competent *E.coli* cells.
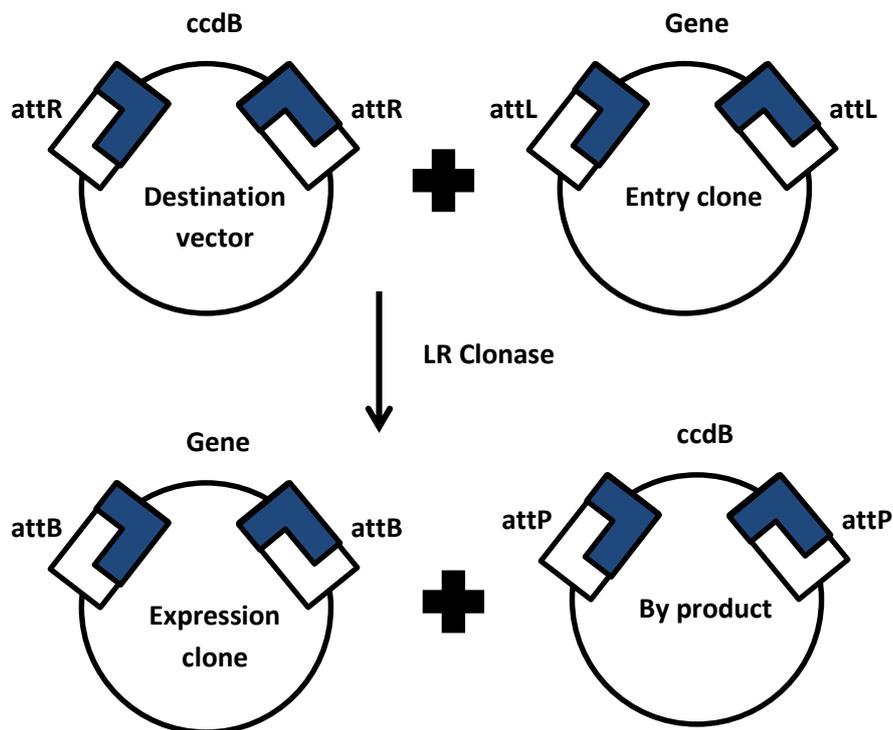
Figure 2.4 Diagram representing the principles of the Gateway LR reaction, generating an expression clone.

2.9.2 Hairpin cloning to knockdown genes

To transiently repress CHO genes, the Hairpin Cloning system from Promega was used. In this system the siRNA are expressed as foldback stem-loop structure which act to suppress expression of a target sequence by stimulating specific degradation of the target mRNA (Zamore, 2001). This is achieved using a vector containing a U1 promoter which allows transcription of the hairpin target sequences. The hairpin insert is formed by two DNA oligonucleotides (see Figure 2.4) which anneal to form the insert; this contains the hairpin siRNA target sequence with sticky ends to facilitate ligation with the vector provided. This vector contains the gene for ampicillin resistance for screening of successful ligation once transformed into *E.coli*. More details on this method are provided in the manufacturers Hairpin cloning manual. Once the cloning is complete, the vectors containing the hairpin sequence were transiently transfected into the stable cell lines expressing GLuc, and GLuc levels determined by western blot.
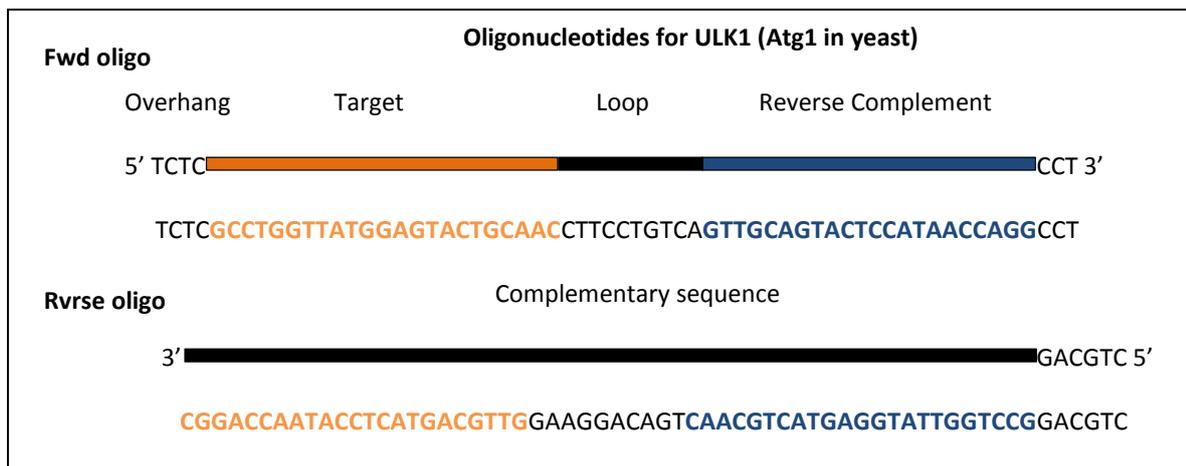
Figure 2.5 Example of oligonucleotide sequences for the hairpin cloning system. A forward and reverse oligonucleotide (which is complementary) is used to knock down ULK1.

Oligonucleotide A is the template strand for cellular RNA polymerase II and should contain an overhang sequence which is complementary to the pGeneClip vectors, completing the U1 promoter sequence. The loop sequence provides flexibility for the RNA hairpin formation. The target sequence forms the double-stranded portion of the hairpin in combination with the reverse complement sequence. Oligonucleotide B is complementary except for the overhang sequence (GACGTC), which contains an additional nucleotide to form a *PST I* restriction site allowing for detection of successful clones.

2.9.3 DNA Quantification

To measure DNA concentration, the BMG Labtech SPECTROstar Nano was used. Using an LVis Plate, 2 µl of sample was loaded and a reading at OD_{260} was measured.

2.9.4 Restriction digest

This reaction uses restriction endonucleases which cut at specific sites on DNA plasmids. Below in Table 2.17 are the details of the method used for this process for one reaction.

Table 2.17   Reagents used in restriction digest. This reaction mixture was incubated at 37°C for 1-2 hours.

Reagents	Volume
10x buffer	2 µl
Restriction enzyme	1 µl
DNA (1 µg in TE pH8)	1 µl
BSA	0.2 µl
Sterile H_2O	Up to 20 µl

2.9.5 Sequencing

To check cloning was correct, purified DNA samples (10 µl of 100 ng/µl) were sent to Source Bioscience Lifesciences for sequencing. The results from this were analysed using ApE plasmid editor.

Chapter 3

Optimisation of a Yeast Model System for Screening Strains with Altered Recombinant Protein Production

3.1 TOR signalling and recombinant protein production

3.1.1 The industrial use of yeast for recombinant protein production

The biopharmaceutical industry is continually growing in terms of the number of protein- based products in the clinic and in development, and now makes up a significant portion of total drug sales, indeed 8 of the top 10 drugs by sales are protein based drugs (Walsh, 2014). In line with the product requirements of recombinant biotherapeutic proteins, production is carried out using a number of different cell based expression systems ranging from micro-organisms to insect, plant and mammalian cell systems. Methods for recombinant protein production are continually developing and evolving, with modifications and optimising of current expression systems being addressed both in academia and industry. For example, investigating and altering biological pathways or processes that are involved in properties that contribute to recombinant protein production with the aim of increasing product quality and yield have been reported (Edros, McDonnell, & Al-Rubeai, 2014; Hamilton & Gerngross, 2007; Lee, Ha, Park, & Lee, 2013). More detail on such approaches is described in the introduction chapter of this thesis.

The results presented in this chapter focus on the use of the budding yeast, *Saccharomyces cerevisiae,* which is one of the most robust and best-characterised experimental systems frequently used for expressing biotherapeutic recombinant proteins, (Hamilton and Gerngross, 2007) and development of a model reporter system of recombinant protein expression. Yeast can be grown to high cell densities in a basic cheap media, facilitating reliable recombinant protein expression and secretion by the cell (De Pourcq *et al.*, 2010). This allows large volumes of heavily glycosylated recombinant protein to be produced. In addition, *S.cerevisiae* poses a very rigid cell wall compared to mammalian cells and can withstand higher levels of stress. They do however possess a disadvantage in that they produce high-mannose glycosylation which is not a suitable replacement for human glycoproteins required for medical products. However, there have been recent advances in this field of engineering which allows yeast to replicate the most essential glycosylation pathways found in mammals (De Pourcq *et al.*, 2010).

In order to achieve the correct/required recombinant protein structure and function, post-translational modifications occur within the secretory pathway. Proteins need to efficiently enter and transit the secretory pathway which requires a signal peptide for entry into and through the endoplasmic reticulum (ER) (Porro *et al.*, 2005). Once in the lumen of the ER, correct folding of a secretory protein is necessary as failure results in its accumulation leading to the activation of the unfolded protein response (UPR) (see chapter 1.2.2 for more detail)(Bravo *et al.*, 2012).

3.1.2 The use of a *Gaussia* luciferase construct as a model secreted recombinant protein system

A vector was developed at Kent that expresses a secreted form of the *Gaussia* luciferase (*GLuc*) protein from the crustacean *Gaussia princeps* within *S. cerevisiae*. GLuc is a small, single chained polypeptide 185 amino acids long which is naturally secreted (Ruecker *et al.*, 2008). In, *Gaussia princeps,* after the synthesis of the luciferase protein, it is packed into secretory vesicles for storage. For the expression of GLuc in mammalian cells, the gene was cloned with a human signal peptide added at the N-terminal end (Knappskog *et al.*, 2007). In yeast, an N-terminal hydrophobic signal peptide is required for efficient secretion of a heterologous protein. Many secretion vectors contain the yeast signal peptide prepro-α-factor from pheromone MFα1 which is secreted by Matα yeast strains during mating (Figure 3.1). The pre-region is 22 amino acids long and is cleaved by the ER membrane-associated signal peptidase. The pro-region contains three N-linked glycosylation sites and is cleaved within the Golgi complex by the Kex2 enzyme at the Lys-Arg site to generate an authentic N-terminus on the secreted protein (Tuite, Clare & Romanos, 1999). This pre-pro peptide has been cloned at the N-terminal of the GLuc gene into the yeast vector pBevy-U.
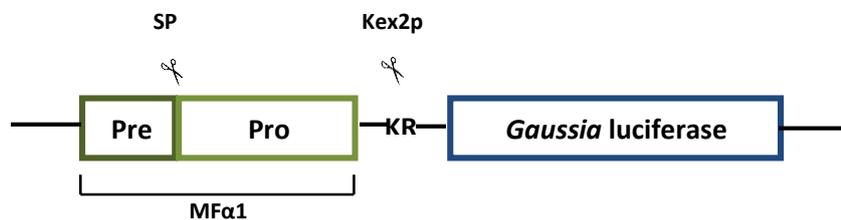
Figure 3.1 Organisation of the prepro-α-factor from *S.cerevisiae* showing cleavage sites by signal peptidase (SP) and Kex2p. The pre-pro region is taken from the yeast mating alpha factor containing a signal sequence.

Like other reporter systems such as *Renilla* luciferase, Gluc uses the substrate coelenterazine and light for bioluminescence (Ruecker et al, 2008) and is reported to possess an increased bioluminescence signal over firefly and *renilla* luciferase (Tannous *et al.*, 2005)*.* This reaction with GLuc is shown schematically in figure 3.2.
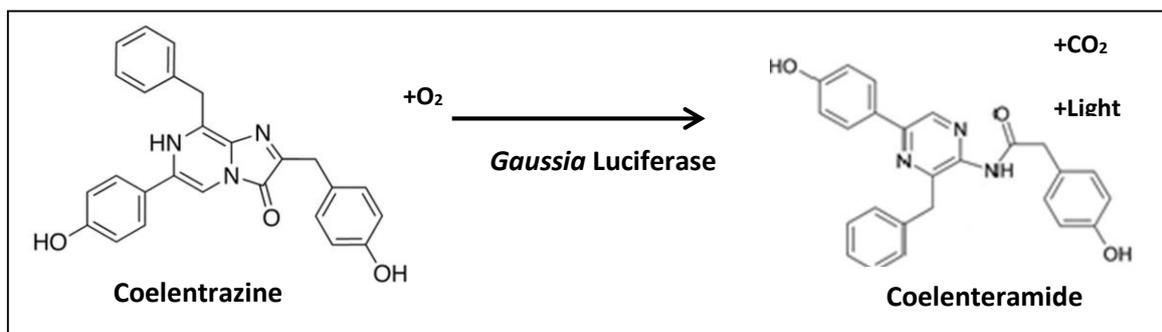
Figure 3.2 Reaction between GLuc and the substrate coelentrazine producing bioluminescence which is detected and quantified. The oxidation of the substrate produces coelenteramide, CO_2 and light.

A further advantage of Gluc as a reporter protein within an expression systems is that it does not require ATP for activity so it can report from cell surroundings as well as from the cell pellet (Tannous, Kim, Fernandez, Weissleder, & Breakefield, 2005). Here, we utilised plasmids containing *GLuc* to investigate secreted recombinant protein production in yeast (Figure 3.1).

Results

3.2 Towards optimisation of a model system for screening TOR mutants from the deletion library

The ultimate aim of the work described in this thesis was to screen TOR and other mutants for the effect of these mutants on cell phenotypes, particularly the ability of the cell to secrete recombinant proteins. As such it was necessary to develop systems that could be used to screen for such effects.

3.2.1 Using a *Gaussia* Luciferase assay as a screen to measure recombinant protein production

3.2.1.1 GLuc bioluminescence and hence production throughout culture

An initial GLuc assay was conducted to examine whether growth phase affected the bioluminescence output over a 25-hour period following inoculation of yeast cells at an OD_{600} nm of 0.3. This was performed using wild type cells grown in minimal media. Samples were analysed in duplicate and readings taken at 3.5, 6.5, 9.5 and 25 h after inoculation. This allowed investigation of bioluminescence at log phase (3.5, 6.5, 9.5 h) and stationary phase (25 h) of growth. The highest readings observed

were taken from cells within log phase growing at their maximal rate. Following this, a decline in bioluminescence was observed as the cells growth rate decreased as they entered the diauxic phase stage of growth. During this stage of growth the yeast had depleted glucose within the media and cells commence ethanol utilisation, a product of fermentation. With ethanol depletion, yeast cells enter stationary phase where the growth plateaus. At this point we observed almost an entire loss in GLuc bioluminescence (Figure 3.3).

A)
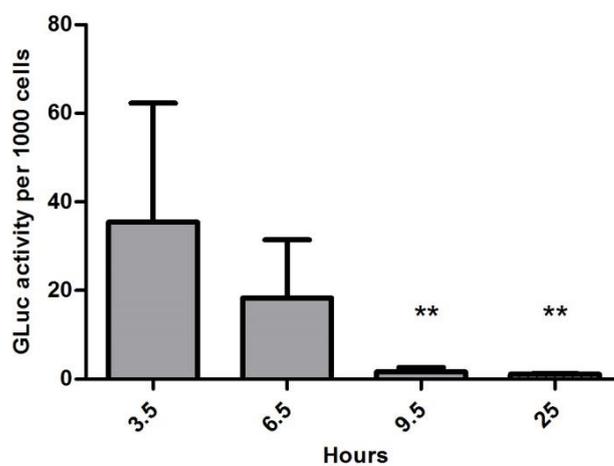
B)
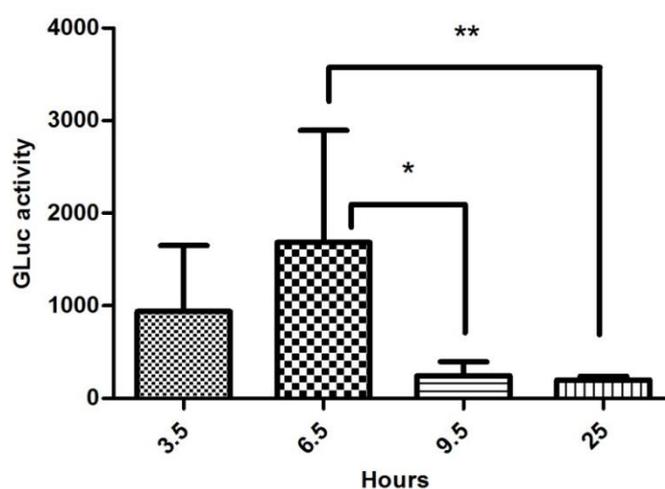
C)

Hours	Cell count/ml
3.5	1060000
6.5	3680000
9.5	5960000
25	7370000

Figure 3.3 GLuc luminescence over a 25 h period as determined at the time points indicated when grown in minimal media. A) GLuc activity per 1000 cells. The highest levels of GLuc were detected 3.5 h after cells were inoculated at an OD_{600} of 0.3 (n=6). B) Raw GLuc activity readings. C) Cell count/ml at each time point. Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test with significance annotated comparing samples at varied times to 3.5 hours (**p= <0.01).

Despite undertaking the time course analysis multiple times and undertaking replicate measurements at each time point, variation in GLuc activity was observed as shown by the large error bars (Figure 3.3).

Figure 3.3B shows how there is an accumulation of GLuc detected between 3.5 and 6.5 hrs, yet there is no further accumulation after 9.5 hrs followed by slightly further decrease in GLuc detected after 25 hrs. This suggests that more GLuc was expressed earlier on in the time course; this was expected as the promoter is known to actively drive expression of GLuc protein during log phase. There is an increase in GLuc detected at 6.5 hrs compared to 3.5 hrs. However, the cell count at 6.5 hrs is more than 3 times higher than at 3.5 hours, yet it shows roughly less than 2 times the GLuc accumulation after 6.5 hrs. This is also seen in Figure 3.3A where GLuc activity per 1000 cells shows higher bioluminescence detected at 3.5 hrs compared to 6.5 hrs. The data from both graphs implies that there is degradation of the protein produced between 3.5 hrs and 6.5 hrs. This would also explain the loss of GLuc after 9.5 hrs where further degradation of the protein would also occur. However, due to inter-replicate error any data produced using this assay makes definitive conclusions challenging. Yet from the data shown here, this suggests that further assays measuring GLuc activity should be done at 3.5 hrs growth.

To determine whether pH changes could lead to differences in luminescence output, cells were grown in media buffered to varying pH values and the growth and Gluc luminescence then assessed. The resulting growth curves are shown in figure 3.4.

A)
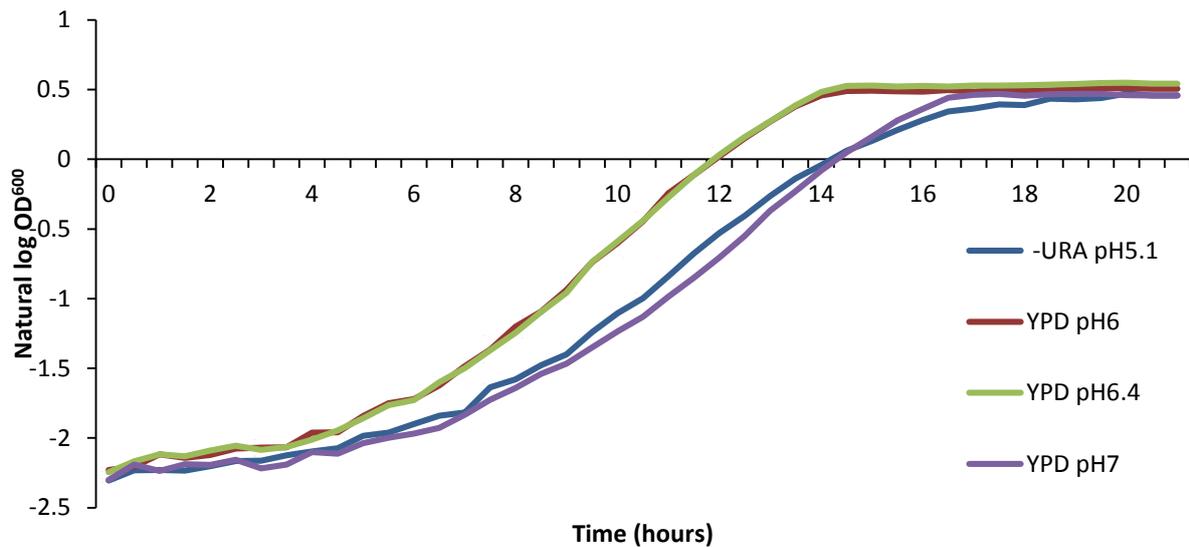
B)
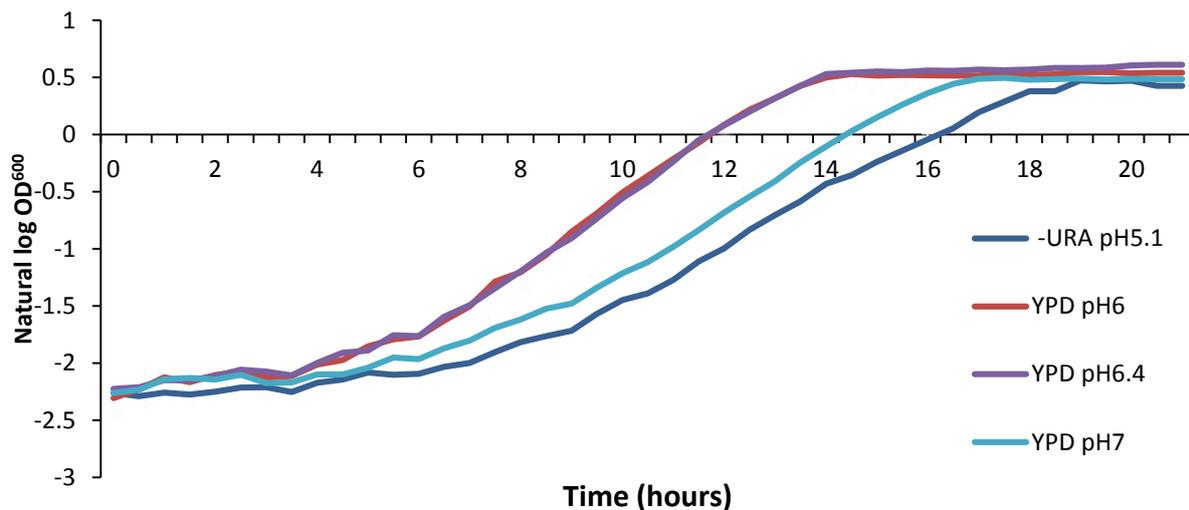
Figure 3.4 Average growth of wild type BY4741 cells in minimal media (pH 5.1) and YPD at varied pH. A) Growth of the wild type expressing the plasmid lacking GLuc. B) Growth of the wild type with the GLuc expressing plasmid. Potassium phosphate buffers were used to get the media to the required pH. Cells were inoculated at an OD_{600} of 0.1 and grown into stationary stage (n=2).

Growth of the wild type with and without the expression plasmid was similar in media at pH 6 and 6.4. However, growth rate was affected in media lacking URA and YPD pH 7 with a longer lag phase (Figure 3.4). As the data suggested that pH had an effect on growth, whether pH also led to differences in Gluc bioluminescence was also investigated. The results are shown in figure 3.5 below.
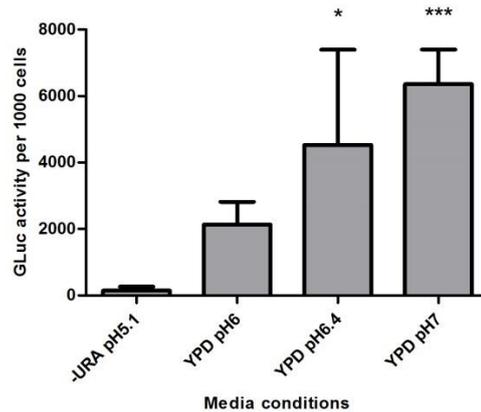
Figure 3.5 GLuc activity at varied media and pH conditions. Cells were grown to an OD_{600} of 0.3 and readings taken after 3.5 hours (n=6). Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test comparing readings in –URA to YPD media (*$P<0.05$, ***$P<0.001$).

Under increased pH media conditions the luminescence was higher suggesting that these conditions were better for secreting GLuc from the cells (Figure 3.5). Upon varying pH conditions, a change in both cell growth and the reproducibility of the Luciferase assay readings was observed. Suitable conditions for this assay were between pH 6.4 and pH 7, as pH 7 gave the highest activity in the Gluc assay but there was better growth at pH 6.4. Cultures were also grown to determine the viability of the cells under these different pH conditions and the results are shown in figure 3.6.
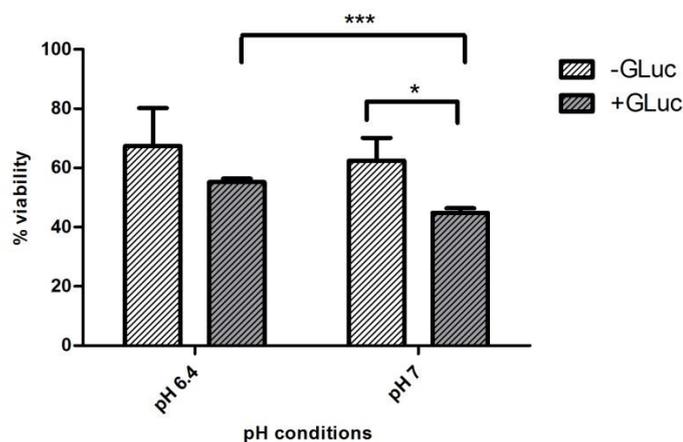
Figure 3.6 Viability assay at pH 6.4 and pH 7 containing a plasmid with/without the *GLuc* gene. 300 cells were plated and colonies counted after 2-3 days (n=3). Error bars represent standard deviation. ANOVA was used to determine statistical significance (*$P<0.05$, ***$P<0.001$).

The results of the viability investigations showed a statistically significant increase (***p<0.001) in viability at pH 6.4 compared to pH 7 expressing GLuc, which is the pH closer to the optimum of standard YPD cells are grown in. However, this particular assay is not entirely accurate with enumerating colony forming units. For example, cell counts may be inaccurate when performed before plating out 300 cells due to cells budding. The cells plated out can also be clumped forming what appears to be only one colony, instead of several.

The GLuc assay was repeated with cells grown in YPD at pH 6.4 which was the optimal pH to encapsulate good cell viability together with sufficient GLuc activity. The results from this investigation are shown in figure 3.7 below.
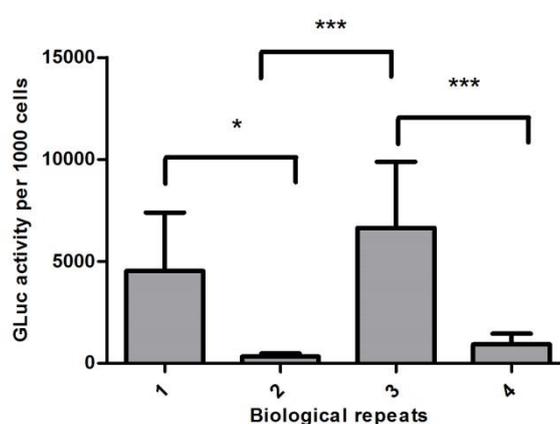
Figure 3.7 Reproducibility test of Gluc assay at pH 6.4 repeated on 4 different days. The bars show bioluminescence in the wild type (n=6). Cells were inoculated at an OD$_{600}$ of 0.3 and grown for 3-4 hours before the assay was performed. Errors bars represent standard deviation, and significance determined using an ANOVA test (*P<0.05, ***P<0.001).

When cells were grown at pH 6.4, high variability was seen between individual days and within each day to a lesser degree (Figure 3.7). A potential source of variability could be where after re-inocculation the following morning, the cells used are at varied phases of growth. If at varied phases of growth it could take some cells longer to get into log phase, thereby effecting GLuc produced as the promoter is switched on. If after the reinoculation the next morning, the cells were left to grow for a further 24hrs and then reinnoculated a second time, hopefully the cells would be in the same phase of growth.

All of the luminescence data showed higher variability for samples than was ideal and therefore as an alternative to using the GLuc activity assay, GLuc protein expression was determined by western blotting with a monoclonal antibody against GLuc protein raised in rabbit.

3.2.2 Measuring the expression of *Gaussia* Luciferase by western blot

GLuc expression in the yeast cells was evaluated by western blotting as an alternative to using the GLuc activity assay to determine if this gave results with less variability. Although this particular method is still only semi-quantitative as when the membrane containing the protein is exposed to the film, this is non-linear, in particular during short exposures. When the film containing is scanned into the computer and quantified using Image J, some of the quality of the autoradiograph may be lost.

The levels of GLuc expression determined from these blots indicated whether strains were low or high recombinant protein producers. Cells were grown in standard YPD (pH 6.6) to a density of $1\text{x}10^7$ cells/ml and protein extracted. Both supernatant and cell pellet samples were taken to analyse how well the cell processes and secretes the GLuc protein and how much was maintained within the cell. GLuc expression detected from the resulting blots, and β-actin from intracellular samples used to normalise data against the cell number, were processed by densitometry using the freeware software Image J. The resulting data is shown in figure 3.8 below.

The expression of GLuc was detected in the pellet and supernatant of the wild type (Figure 3.8). There was a more intense band of GLuc retained in the cell (58.5%) whilst a number of other unidentified bands were also observed. These unidentified bands of higher molecular weight could be due to incorrectly proccessed forms of GLuc with varied modifications causing a shift in size such as glycosylation or other post translational modifications. As these are only observed in the pellet, one of these at least could be due to unprocessed GLuc where the signal sequence has not been cleaved. Quantification of the blot images confirmed that these results were more reproducible than the GLuc activity assays previously described. Experiments were kept consistent to avoid any artefacts and a chemical lysis buffer used for extraction that has previously been shown to give the highest extraction efficiency for more quantitative data (von der Haar, 2007).

A)

B)
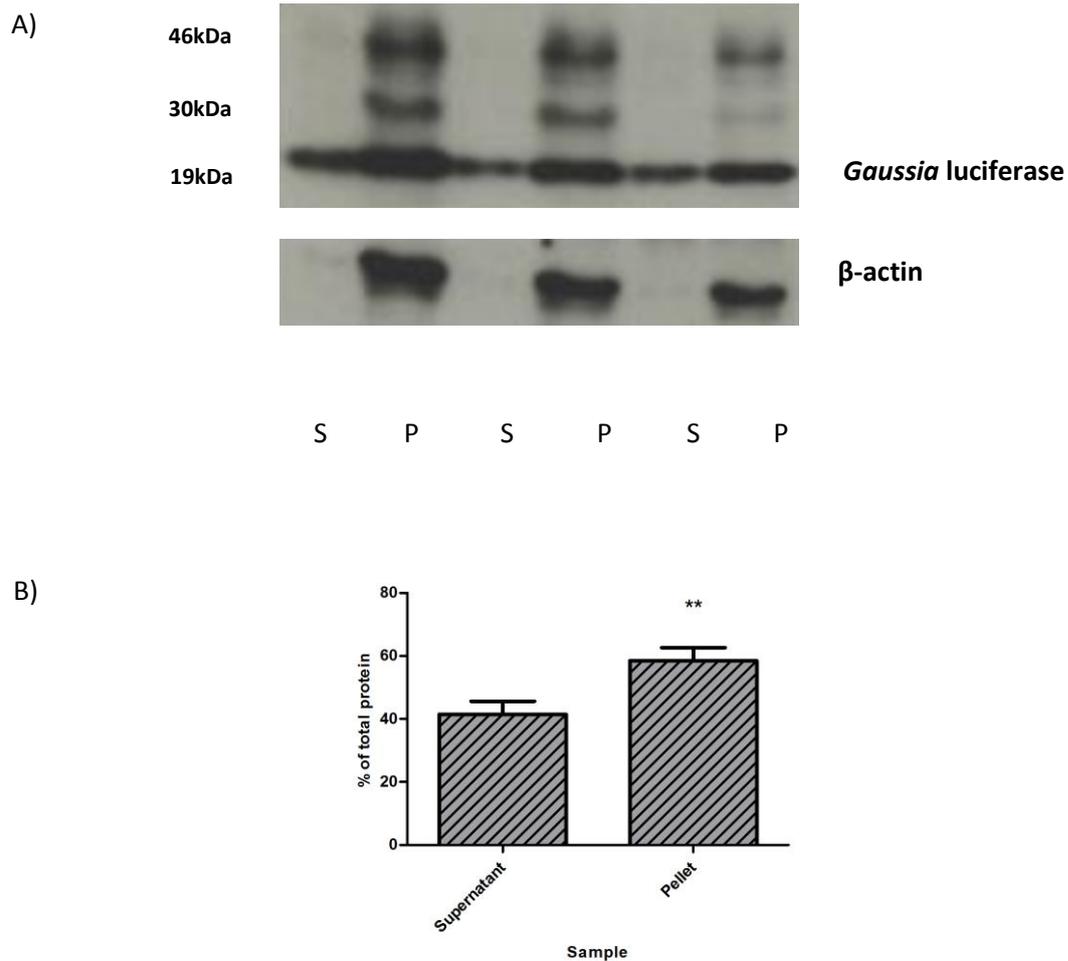
Figure 3.8 Expression of GLuc and β-actin in the wild type. A) GLuc protein levels were analysed in biological triplicate. S represents supernatant, P represent pellet sample. B) Quantification of GLuc expression in the supernatant and pellet. Cells were grown overnight in minimal media and then inoculated at an OD_{600} of 0.3 in YPD and left to grow for 3-4 hours. Errors bars represent standard deviation and statistical analysis performed using a two-tailed t-test (p=0.0075).

3.2.3 Testing Killer Toxin secretion

3.2.3.1 Introduction to the Killer Toxin

Another method that can be used to monitor protein secretion in yeast utilises the yeast killer toxin. In this assay, the cell secretes the toxin K1. Yeast which produce this toxin have an advantage over others during times of scarce nutrients. This toxin is conferred by a cytoplasmically inherited double-stranded RNA (dsRNA) virus found in yeast and fungal species. However, the killer phenotype in some cases is due to the presence of dsDNA plasmids or the toxin is chromosomally encoded (Schmitt and

Breinig, 2006b). In yeast, killer strains have been classified into three major groups depending on the toxin secreted and the component that gives these cells immunity to their own toxin.

The three killer strain groups are K1, K2 and K28 and are associated with the presence of the inherited dsRNA satellite virus (ScV-M1, ScV-M2 or ScV-M28 which are about 1.8 kb) which is stably maintained and replicated in the cytoplasm and relies on the presence of the L-A helper virus (4.6 kb)(Schmitt and Tipper, 1995; Schmitt and Breinig, 2002). Both these dsRNA genomes are separately encapsulated into virus-like particles (VLPs) and are spread via mating or heterokaryon formation (co-existence of two or more genetically different nuclei in the cytoplasm). The L-A virus contains two ORF's, Gag, the major capsid protein for the encapsulation and viral particle structure and Pol, the RNA-dependent polymerase. Conversely, the genome for the M virus only contains the ORF coding for the preprotoxin (pptox) which is the unprocessed precursor of the mature secreted killer toxin. It is this ORF which is required for immunity though this is not currently well understood (Schmitt and Breinig, 2006b; Esser, 2009).

3.2.3.2 Processing of the toxin

The preprotoxin (pptox) contains the N-terminal signal sequence which is the precursor required for the pptox import into the ER lumen which is followed by the subunits α and β of the mature toxin (Figure 3.9). These mature toxin subunits are separated by the γ subunit which is N-glycosylated. After the signal sequence is cleaved off, further processing is undertaken through the secretory pathway giving a biologically active α/β heterodimer. It is in the late Golgi where the γ subunit is removed by Kexp and the C terminal of β subunit is cleaved by Kex1p. The mature protein is then secreted as an α/β heterodimer with the subunits covalently linked by disulphide bonds (Schmitt and Breinig, 2006b).
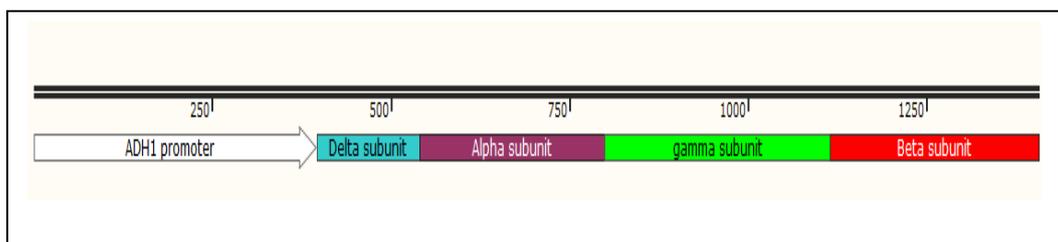
Figure 3.9 Schematic of the four subunits forming the killer toxin gene, K1 with the promoter ADH1. The delta subunit acts as a signal sequence to the ER necessary for secretion.

3.2.3.3 Killer toxin action

Different Killer toxins are capable of killing sensitive yeast strains through a range of receptor-mediated two step processes The first is a fast and energy dependent binding to a toxin receptor in the cell wall. In K1, the receptor is β-1,6-D-glucan. Targeted strains can become toxin resistant by chromosomal mutations in any of the genes involved in structure and/or biosynthesis of the yeast cell wall components. Toxin resistant genes *kre* (killer resistant) have been found in K1. Another mode of action is toxin translocation and interaction with a secondary membrane receptor on the cytoplasmic membrane, Kre1p. After the toxin reaches the cytoplasmic membrane, it exerts its damaging effect by ion channel formation and disruption of cytoplasmic membrane function (Schmitt and Breinig, 2002).

3.2.3.4 Toxin immunity

In the K1 killer toxin, the mode of immunity is currently unknown but it is speculated that the toxin precursor can act as a competitive inhibitor of the mature toxin by saturation or by eliminating the plasma membrane receptor that normally mediates toxicity. Δkex2 mutants would lack the ability to process pptox becoming an immune non-killer yeast strain. Another possibility is that the γ subunit can not only act as an intramolecular chaperone ensuring proper secretory pptox processing but also provide a masking function by protecting the membranes of toxin producing cells against damage as the α subunit has hydrophobic components (Schmitt and Breinig, 2006b).

3.2.3.5 Killer toxin assay

In the killer toxin assay used in this study, K1 was used in a PVT100U plasmid and expressed with an *AHD1* promoter. This was transformed into the wild type creating a killer strain, and the sensitive strain S7 was used. Cells were spotted onto a lawn of the sensitive strain in a series of dilutions. The first spot shows the control strain without the killer toxin (Figure 3.10).

Control **WT+K1** **Increasing cell concentration**
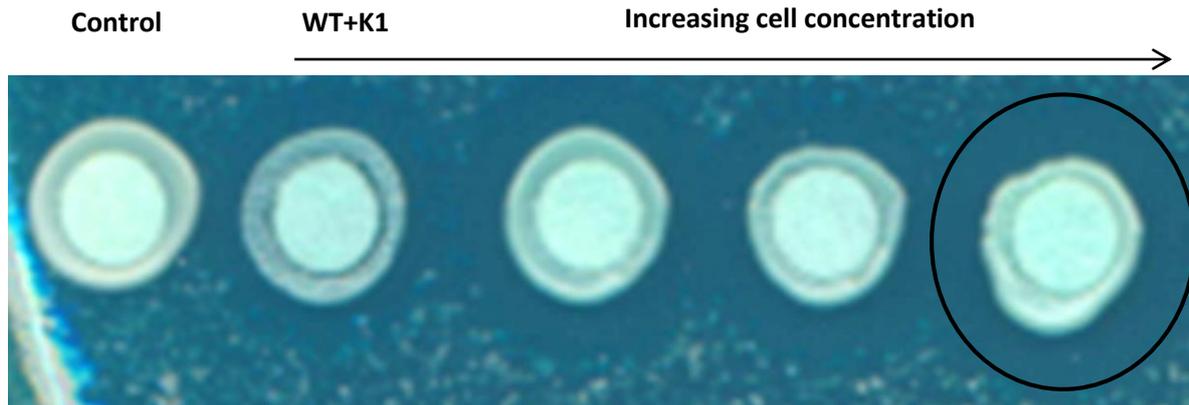
Figure 3.10 Strains expressing the toxin on agar plates containing methylene blue. Strains are spotted on filter paper in a dilution series and left to grow for 2 days killing the surrounding sensitive strain (1×10^5 cells were plated). This kill zone (indicated on the last spot) was measured in Image J for quantification with image contrast enhanced by 0.1%. Right to left dilutions were performed in the killer strain with a 5-fold, 2.5-fold and 2-fold dilution respectively.

There was a clear kill zone surrounding the killer strain made visible through methylene blue when the assay was undertaken (Figure 3.10). The top agar containing the sensitive strain had to be at a pH of 4.8 for their cytotoxic activity to be achieved (Fink and Styles, 1972; Santos and Marquina, 2004).

3.2.3.6 Low pH effects protein secretion

As the killer toxin assay is conducted at a low pH, we were interested in testing how this condition affects the protein production of GLuc. GLuc expression was measured and compared between the wild type at pH 6.6 and pH 4.8 by western blot (Figure 3.11).
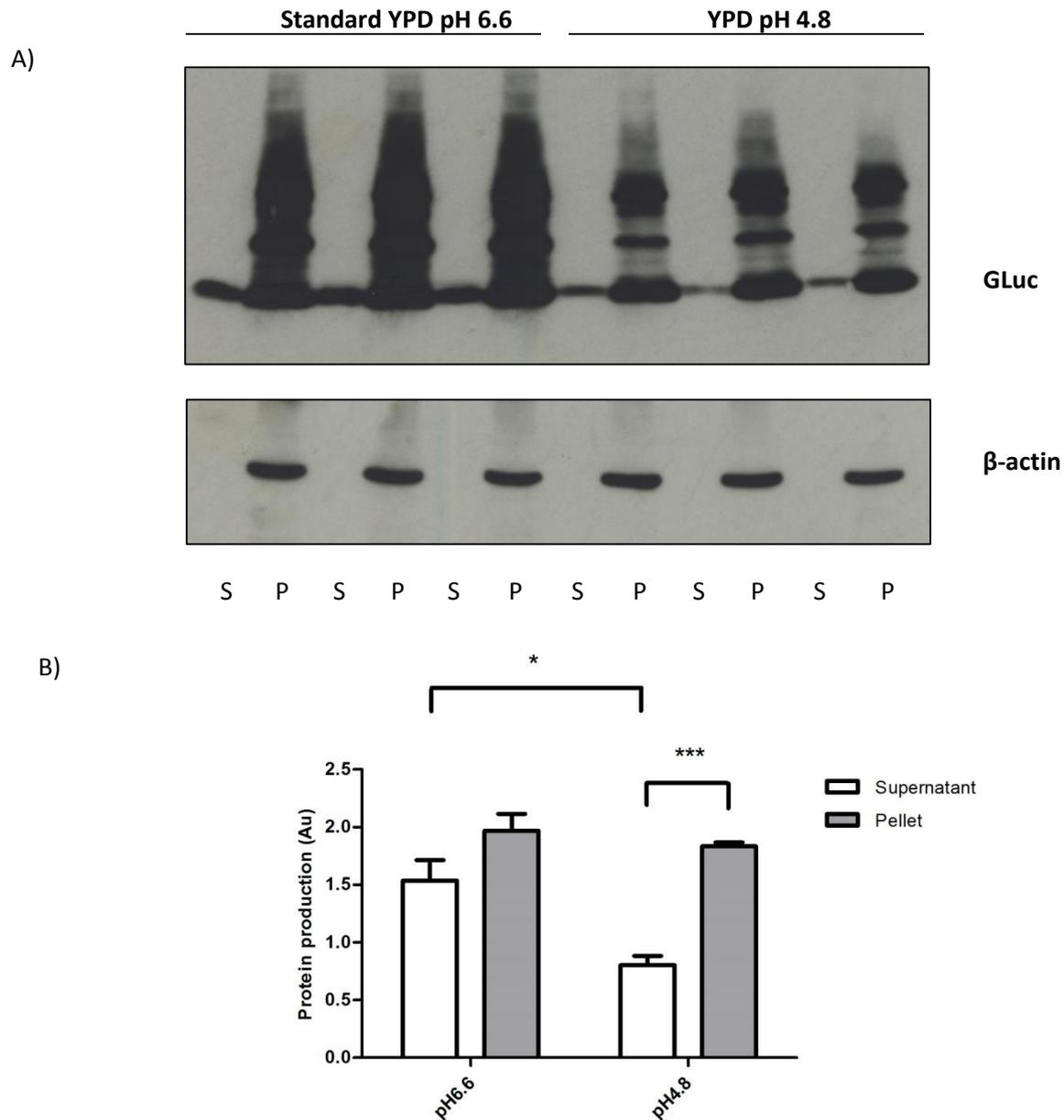
Figure 3.11 pH test on Gluc expression in wild type at pH 6.6 (standard YPD) and pH4.8. A) Western blots with GLuc and β-actin showing expression in supernatant (S) and pellet (P) (n=3). B) Quantification of the blots show lower pH has a negative impact on GLuc secretion. Cells were grown overnight in minimal media and then inoculated at an OD_{600} of 0.3 in YPD and left to grow for 3-4 hours. Errors bars represent standard deviation. ANOVA was used ***$P<0.001$. Low pH has a statistically significant effect on secretion when comparing pH6.6 and pH4.8 p=0.0190 with the t-test).

The results indicated that a reduction in pH of growth media led to a statistically significant decrease in total protein production but there was also a higher percentage of protein trapped within the cell (Figure 3.11).

3.3 Discussion

This chapter describes the development of various assays and the techniques behind them used to find a suitable screen to investigate how changes in a yeast expression system affect recombinant protein production. This was important as the systems and assays developed were then to be used throughout the remainder of the study.

3.3.1 The GLuc activity assay

The GLuc activity assay proved difficult to work with, despite trying to retain consistency in preparation of cells and readings taken. The activity as determined by luminescence was variable, even for replicate measurements of the same sample making the use of the assay limited. An alternate option could have been to use an injector so the readings were taken immediately. The other issue was comparing average readings between days, presenting wide variation. During fermentation, yeast produce acetate while depleting nutrients in the media causing changes to the surrounding pH which appeared to have an effect on luminescence output. Cells were originally grown in minimal media for selection of the plasmid with a low pH. However, although the GLuc assay is functional over a broad pH, the minimal media is far from the optimal pH of 7.7 (Tannous *et al.*, 2005). After 9.5 hours, where cells are no longer doubling, there was almost no GLuc detected (Figure 3.4). This was expected due to the high expression of the plasmid during log phase as a GPD promoter (glycolytic pathway) is used; yet could also be due to the accumulation of proteases later on in post diauxic shift.

Growth analysis had shown that growth in minimal media at pH 5.1 increased lag phase time; the lack of nutrients explained this phenotype. Wiles et al. (2005) reported that GLuc bioluminescence is unaffected by exposure to low pH, despite this causing stress on cells. YPD at pH 7 demonstrated a growth defect similar to that of the minimal media, yet the highest GLuc activity was detected in this condition. pH 6.4 appeared to be the optimum pH for cell growth, as this had the second highest GLuc activity detected with a higher percent of viable cells compared to growth at pH 7. Perhaps proteases released are inactive at this pH, resulting in higher luciferase activity. GLuc is reported to be highly stable at a wide range of pH values so it is more likely to have been the substrate coelenterazine being affected.

3.3.2 Using western blotting to determine GLuc expression

Regardless of finding the best pH conditions, results remained inconsistent and the GLuc activity assay could not be used as a screening method and as such western blots were utilised as an alternative. This method also gave information of how well the cell was secreting against the amount of material found intracellularly (Figure 3.8).

Western blots still have some problems with variability however, such as incomplete protein extraction or inefficient transfer of the protein to the PVDF membrane. Due to large numbers of strains, membranes containing the protein extracts could not all be exposed at the same time, introducing more room for error. Further, this analysis informs on the total amount of protein present but not on activity (as given by the assay) and hence it is possible that changes to the amount of protein made may not reflect changes in active protein produced.

3.3.3 The killer toxin assay

The pre-pro sequence helped increase protein production as the cells under stress try expressing the pheromone. As the data is specific to this construct we also wanted to use the Killer toxin assay as another screen. This viral model system has been used for a number of applications such as analysing specific aspects of yeast virology, used in food and fermentation industries but also in the field of recombinant DNA technology. The secretion and processing signal derived from ScV-M28 killer virus in *S.cerevisiae* has been used in *S.pombe* to target foreign proteins for secretion (Schmitt and Breinig, 2002). The killer toxin assay is fast, cheap and highly reproducible, and was an ideal alternative screening method alongside the western blots for GLuc. As the Killer toxin used a different promoter and signal sequence, the protein was predicted to have different secretion compared to strains in the GLuc western blots.

The killer toxin's cytotoxic activity functions at pH 4.8, and therefore the assay was performed in this condition. As it is known that pH has no effect on the stability of GLuc (Tannous *et al.*, 2005; Maguire *et al.*, 2009), we looked at the effect of growing the cells at pH 4.8 and its consequence on protein expression detected by western blots of GLuc. We demonstrated the lower pH conditions caused a loss in GLuc expression (Figure 3.11). Our knowledge about the molecular basis for pH stress is limited and have no further data suggesting what is causing this result (Mattanovich *et al.*, 2004).

3.3.4 Conclusion

Given the results of the experiments presented here, it was decided to use western blotting as an alternative to the GLuc assay and the killer toxin assay as screening tools for investigations. To measure protein expression samples were collected when yeast cells were in log phase with growth in standard YPD at pH 6.6. The killer toxin assay meanwhile continued to be performed in a dilution series at pH 4.8. These assays were then used to investigated and identify novel ways of improving recombinant protein production in the following chapters.

Chapter 4

The Role of the Actin Binding Protein Cofilin in Stress Signalling and Recombinant Protein Production

4.1 Introduction to the Cofilin

4.1.1 New roles for cofilin in stress response mechanisms

Actin dynamics play an essential role in many processes such as cell motility and neural pathfinding, cell polarity, membrane dynamics, and cell polarity (Bernstein *et al.*, 2006). Cofilin is an essential gene with a central role in regulating the actin cytoskeleton. Members of the cofilin family exhibit a high level of its structural and functional conservation in lower and higher eukaryotes (Yahara *et al.*, 1996). Cofilin is an actin binding protein that associates with ADP-bound actin found at the minus, or pointed, end of actin filaments. Upon binding cofilin helps to stabilize a twisted form of the actin filament which promotes fragmentation and release of actin monomers (Bamburg and Wiggan, 2002) in a pH dependent manner. This pH-dependent function may be physiologically significant as cystolic alkalisation is induced by serum or growth factors, which at a pH higher than 7.3, enhance the ability of cofilin to depolymerise F-actin (Yahara *et al.*, 1996). At a pH lower than 7.3, the ability of cofilin to bind and sever F-actin is reduced (Bernstein and Bamburg, 2010). In mammalian cells inactivation of cofilin is mediated by phosphorylation at serine3 by kinases LIMK or TESK then reactivated by the phosphatase slingshot (for more detail see figure 1.4 in chapter 1) (DesMarais *et al.*, 2005). Alignment with cofilin in S.*cerevisiae* show serine 4 corresponding to serine 3 in vertebrates, however phospho-regulation has not yet been shown in yeast (Mizuno, 2013). Ser4 was replaced with alanine intended to encode constitutively unphosphorylated cofilin, however had very little effect. Mutation in this serine site to glutamine creates a lethal phenotype suggesting that phosphorylation would disrupt the interaction between cofilin and f-actin(Lappalainen *et al.*, 1997).

Besides its actin binding role, Cofilin is involved in the control of a number of signalling pathways and has been shown to contribute to the control of mitochondrial function when a cell is under stress (Bernstein *et al*., 2006). For example it has been shown that cofilin plays a role in apoptosis when active (dephosphorylated) and targets the mitochondria (Chua *et al.*, 2003) . The oxidant, taurine chloromine (TnCL) generated by neutrophils cause cofilin to lose its affinity for actin and translocate to the mitochondria nucleus where upon it promotes the permeabilisation of the outer membrane and release of cytochrome C (Klamt *et al.*, 2009). Another example of how cofilin is involved in stress responses has been found in neurones, whereby ATP depletion induces cofilin to assemble into aggregates, termed rods. The formation of ADF/cofilin rods has been implicated in mitochondrial dysfunction and may play a role in neurological diseases such as Alzheimer's disease (Davis *et al.*, 2011).

New functional surfaces of cofilin which lie outside of the actin binding regions and which link mitochondrial function and stress responses have been identified in *S. cerevisiae* (Kotiadis *et al.*, 2012). Alterations to cofilin residues outside of the actin-binding region produced a dramatic increase in mitochondrial function causing increased resistance to drugs such as azoles due to the upregulation of a battery of ABC transporters from retrograde signalling (Kotiadis *et al.*, 2012). Microarray analysis suggested that cofilin may control respiratory function post-transcriptionally (Kotiadis *et al.*, 2012). Cofilin is also regulated by inhibition through binding to the phosphoinositides such as phosphatidylinosital 4,5-bisphosphate (PIP2) which are known to mediate cofilin-actin interactions (Ojala *et al.*, 2001). The Gourlay lab discovered that some of the residues mutated in their study which were implicated in PIP2 binding displayed elevated levels of respiration coming to the conclusion that PIP2 may be involved in influencing mitochondrial function via PIP2 interactions (Kotiadis *et al.*, 2012).

ER homeostasis is essential for cell function in protein folding but also in survival. ER stress is where the folding capacity of the organelle is overwhelmed due to increased protein load or disruption to its ability to fold proteins (Vannuvel *et al.*, 2013). Prolonged ER stress eventually leads to apoptosis, where the mitochondria releases cytochrome C and other apoptotic factors such as AIF1 (apoptotic inducing factor 1)(Franklin-Tong and Gourlay, 2008).

In yeast, mitochondria have a mechanical link to the ER via a protein complex identified as the ERMES (ER-mitochondria encounter structure) junction. This junction allows communication to occur between the organelles controlling membrane biosynthesis, protein import, motility and genome replication (Kornmann and Walter, 2010). Communication between the ER and mitochondria also occurs via calcium signalling which plays an important role in ER quality control and proper folding of functional proteins as there are a number of calcium binding chaperones such as calreticulin (Bravo *et al.*, 2012; Vannuvel *et al.*, 2013). Examples where calcium exchanges between the two organelles is important include Krebs cycle dehydrogenases stimulation and calcium dependent ER chaperones (Bravo *et al.*, 2012).

Bravo *et al* (2011) found that during acute ER stress, there was an increase in ER-mitochondrial Ca^{2+} transfer as mitochondria redistributed to the perinuclear zone. This increased the metabolic state of mitochondria as Ca^{2+} increases its membrane potential stimulating oxidative phosphorylation and oxygen consumption, creating more ATP. This enhancement in mitochondrial metabolism is an adaptive response increasing the cell's bioenergetics supply to ER damage by misfolded proteins (Bravo *et al.*, 2011). It is not just ER stress leading to mitochondrial dysfunction and apoptosis, it has also been suggested that mitochondrial dysfunction due to free Ca^{2+} increases levels in the ER Ca^{2a} disrupting homeostasis in the ER leading the ER stress(Lim *et al.*, 2009). As it has been shown that this

ER-mitochondrial coupling is important during early ER stress, our rationale is that as cofilin mutant alleles have increased mitochondrial biogenesis, this may improve rP yields as these strains will be better adapted to increased stress of producing rP in cells with increased coupling between the mitochondria and ER.

4.1.2 Characterisation of mutant cofilin alleles

To determine whether cofilin may play a role in the ability of cells to produce RP we made use of a library of yeast strains expressing mutant alleles of COF1 as the sole source (Lappalainen *et al.*, 1997). In this collection of strains surface charged residues of cofilin have been mutated to alanine, (Figure 4.1) (Lappalainen *et al.*, 1997). These subtle changes have been shown to have an effect on activity and quality of mitochondrial function and have been separated into three classes (Kotiadis *et al.*, 2012).

A)

B)

C)

D)
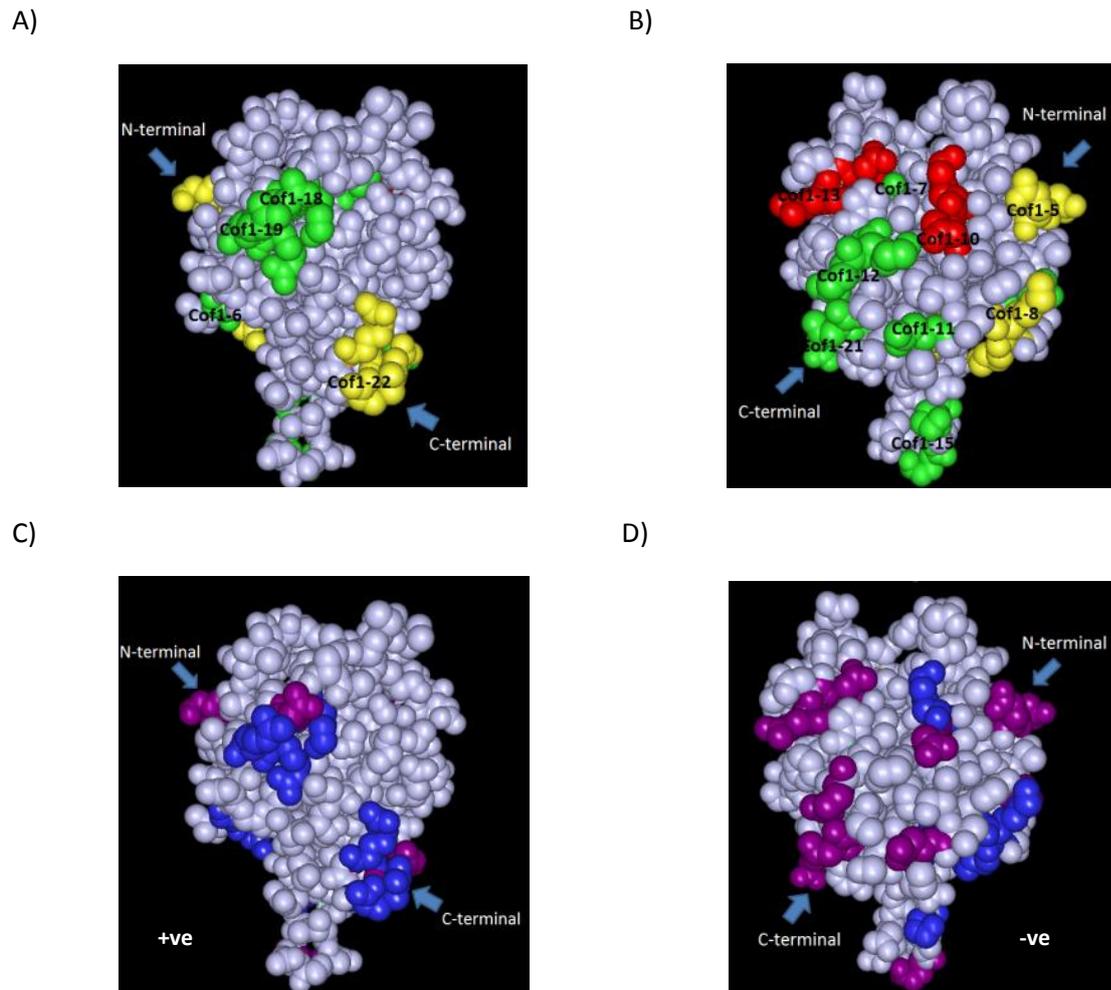
Figure 4.1 Space filling model of the cofilin crystal structure. A) and B) Residues involved in actin binding are indicated in red (essential), yellow (non-essential) and green (indicates residues which are not involved in actin binding) (Kotiadis *et al.*, 2012). C) Positive charged residues are indicated by blue and D) negative charged residues are indicated purple. Crystal structure manipulated through Protein workshop software on Protein data Bank (PDB) ID:1QPV. (http://www.rcsb.org/pdb/home/home.do).

Table 4.1 Table of altered cofilin alleles with growth and actin organisation phenotypes. Adapted from(Lappalainen *et al.*, 1997).

Mutant allele	Amino acid substitution	Growth	Actin organisation
cof1-4	S4A	Wild type	Wild type
cof1-5	D10A,E11A	Temperature sensitive	Minor depolymerisation defects
cof1-6	D18A,K20A	Wild type	Wild type
cof1-7	C62A	Wild type	Wild type
cof1-8	K23A, K24A, K26A	Temperature sensitive	Severely altered
cof1-10	K42A,E43A	Wild type	Wild type
cof1-11	D47A,D51A	Wild type	Wild type
cof1-12	E55A,K56A	Wild type	Wild type
cof1-13	E59A,D61A	Wild type	Wild type
cof1-15	E77A,K79A	Wild type	Wild type
cof1-18	K105A,D106A	Wild type	Wild type
cof1-19	R109A,R110A	Wild type	Wild type
cof1-21	D103A	Wild type	Wild type
cof1-22	E134A,R135A,R138A	Temperature sensitive	F-actin binding and depolymerisation issues

Previous studies from the Gourlay lab split all mutated cofilin alleles into three classes of yeast strains. Class I contains *cof1-4, cof1-6, cof1-7, cof1-11, cof1-12, cof1-15, cof1-18, cof1-19* and *cof1-21.* Class I holds the majority of the cofilin mutant alleles whose expression leads to enhanced mitochondrial biogenesis, elevated levels of respiration and increased expression of integral membrane drug pump proteins (Kotiadis et al 2012), yet exhibit wild type growth rates and actin organisation (see table 4.1). Class II alleles include mutants expressing alleles *cof1-10* and *cof1-13.* whcih display impaired actin regulatory function as these amino acid alterations fall within the actin regulatory region (Figure 4.1). In line with actin playing a role in mitochondria integrity in yeast cells expressing class II alleles fail to respire (Kotiadis *et al.*, 2012). Cell expressing mutant alleles *cof1-5* and *cof1-22* from class III display temperature sensitive growth and exhibit an altered actin cytoskeletal morphology caused by a decrease in F-actin depolymerisation (more dramatic in cells expressing allele *cof1-22*). Expression of

alleles in classes II and III possess mitochondria which appear large and fragmented, and do not respond to signals that promote biogenesis during the diauxic shift phase of growth. Class II mutants display increased reactive oxygen species (ROS) levels that occur as a result of the constitutive activation of Ras2 signalling (CW Gourlay and Ayscough, 2005). The strain expressing allele *cof1-8* has not had the mitochondrial morphology identified, but was temperature sensitive in growth and has severely altered actin organisation placing it in class III.

Evidence from the lab of Campbell Gourlay suggested that Cofilin may also regulate aspects of signals within the Ras/cAMP/PKA (Kotiadis *et al.*, 2012), TOR and MAP kinase transduction cascades (unpublished data). We sought to determine the extent of cofilin's role in the control of cell signalling processes with a view to understanding how it may influence cell behaviour. Of particular interest with regards to RP production was the prospect that increased levels of mitochondrial biogenesis may enhance a cell's capacity for the manufacture of secreted proteins.

Results

4.2 The effect of Cofilin mutations on recombinant protein production

It has been observed that many strains expressing cofilin mutant alleles have increased mitochondrial function and altered cell signalling (Kotiadis *et al.*, 2012). Initially, we sought to determine whether severe mitochondrial dysfunction had an impact upon rP production in yeast cells. To achieve this we used ethidium bromide to generate stains lacking mtDNA as this inhibits the replication and transcription of mtDNA (Desjardins *et al.*, 1985; Armand *et al.*, 2004). mtDNA encodes for seven essential components of the electron transport chain needed for complex III (COB gene), complex IV (cytochrome oxidase gene COX1,COX2 and COX3) and complex V (ATP6,ATP8 and ATP9). Ethidium bromide treated cells , known as petites, or $\rho0$ cells therefore rely on growth by fermentation due to the deficiency of the respiratory chain and cannot grow on non-fermentable carbon sources (Deken, 1966; King *et al.*, 2014). Using the GLuc construct described in chapter 3, we tested recombinant protein production by using Western blotting to determine levels of protein secreted or trapped within the cell. Strains expressing *cof1-4, cof1-18* and *cof1-21* alleles from class I, the *cof1*-10 allele from class II and the *cof1-22* allele from class III were selected with varied mitochondrial function (Figure 4.3).

A)
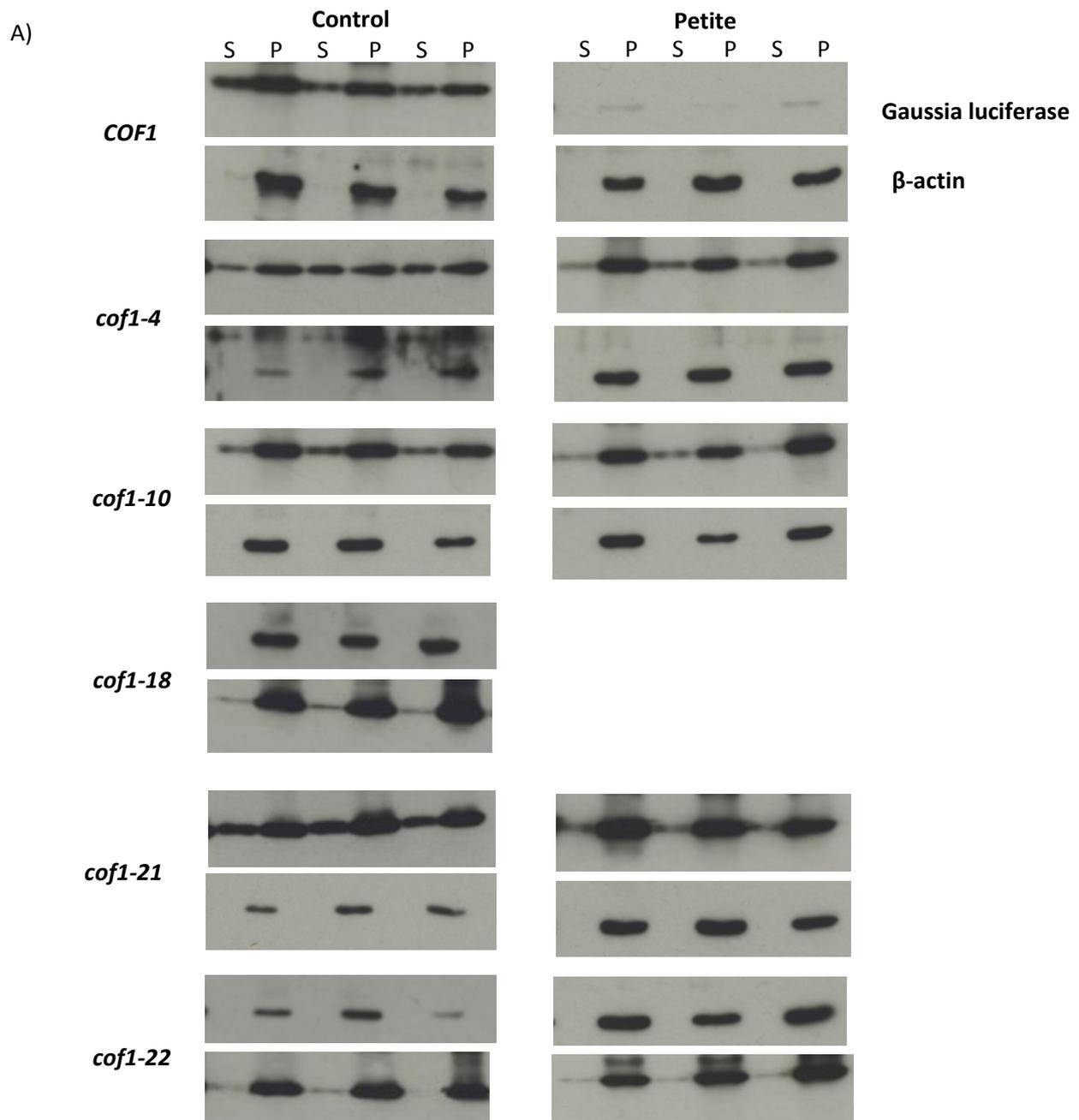
Figure 4.3.1 Expression of GLuc in strains expressing cofilin mutant alleles *cof1-4, cof1-10, cof1-21, cof1-22* and their respective petite strains (ρ0). A) Blots of GLuc expression in mutants and petite strains (no mitochondrial DNA) and actin expression as a loading control. Expression detected in supernatant (S) and pellet (P). Cells were grown overnight in selective media and re-inoculated in standard YPD at OD_{600} 0.1 and grown to the cell count 1×10^7. Protein was extracted from the pellet using the yeast whole cell quantitative method and supernatant treated with 2x sample buffer (n=3). Actin was detected in these strains and used to normalise data.

A)

B)

C)
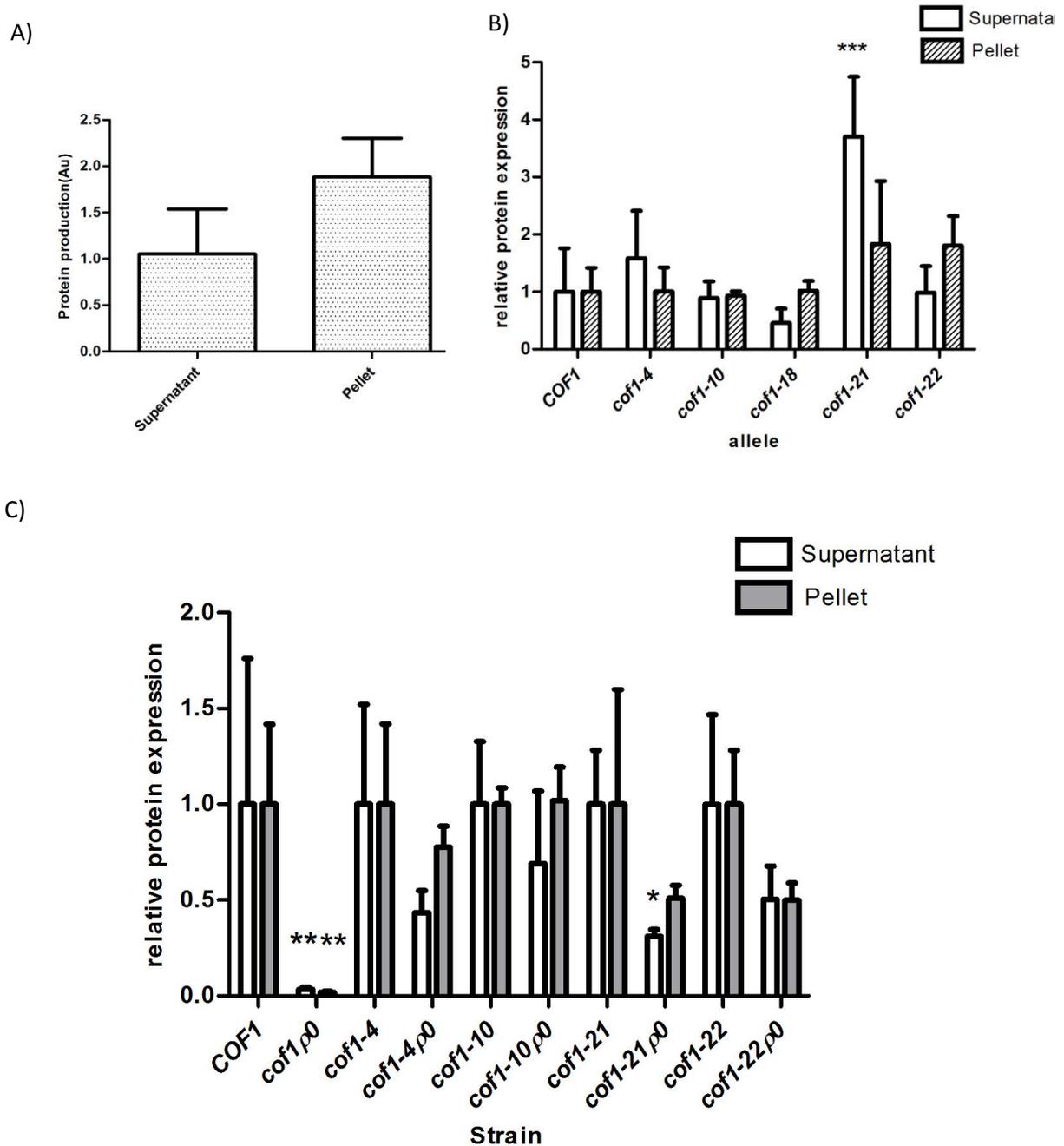
Figure 4.3.2 Quantified expression of GLuc in strains expressing cofilin mutant alleles *cof1-4, cof1-10, cof1-21, cof1-22* and their respective petite strains (ρ0). A) Quantification of wild type strain. B) GLuc detected in supernatant and pellet in the wild type relative to cofilin mutant alleles. C) Quantification of GLuc expression in all mutants relative to their respective petite strain using the free software Image J. Cells were grown overnight in selective media and re-inoculated in standard YPD at OD_{600} 0.1 and grown to the cell count 1×10^7. Protein was extracted from the pellet using the yeast whole cell quantitative method and supernatant treated with 2x sample buffer (n=3). Actin was detected in these strains and used to normalise data. Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test (*P<0.5, **P<0.01, ***P<0.001).

When comparing GLuc protein found in the supernatant and pellet in the wild type, we observed that roughly a third of the protein was secreted (Figure 4.3.2A). In comparison to the wild type, the mutants expressing the alleles *cof1-4, cof1-10*, *cof1-18* and *cof1-22* demonstrated no significant change to rP production, however *cof1-21* exhibited a large increase in secreted Gluc (Figure 4.3.2B).

Another striking result was the outcome of removing mitochondrial DNA from wild type cells. In this case we found an almost total loss of GLuc production (Figure 4.3.1). In contrast, all petite strains expressing the mutant alleles *cof1-4, cof1-10, cof1-21, cof1-22* exhibited higher GLuc expression when compared to the wild type petite strain (Figure 4.3.2C). In petite strains expressing *cof1-4, cof1-21,* and *cof1-22* alleles compared to their respective control strains (with mtDNA), we found removal their mtDNA caused a 50% or more loss in secretion. However, only mutant allele *cof1-21* petite showed a statistical loss (I believe this is because of the large error bars).

In all cases (with the exception of mutant allele *cof1-10*) removal of mitochondrial DNA led to a reduction in rP secretion. However, all cofilin mutants displayed higher GLuc production when mtDNA was absent compared to the wild type petite strain.

The Gluc construct contains the mating alpha factor signal sequence needed for secretion and mating is controlled by MAPK signalling. Therefore, strains with altered MAPK may have an effect on how GLuc is expressed and secreted when associated with the alpha factor signal sequence. We were interested in investigating how well the cofilin mutant strains secreted rP with a different signal sequence. Strains expressing Cofilin mutant alleles were therefore transformed with a plasmid containing the killer toxin gene encoding for the subunits involved in the killer toxin cytotoxicity and immunity (Schmitt and Breinig, 2006a). These strains were spotted onto agar plates with a top layer of ¼ YPD agar, containing a strain known to be sensitive to the effects of secreted killer toxin called S7. When the killer toxin is secreted in this assay it leads to inhibition of growth in the sensitive strain, creating a zone of clearance, or a kill zone (Figure 4.4). The kill zone can be measured and used as an assessment of killer toxin secretion levels (see chapter 3 figure 3.9 for more details). Using this assay, we compared the secretion capabilities of the wild type to strains lacking mtDNA (Figure 4.4A) and to strains expressing the cofilin mutant alleles *cof1-4,cof1-10*, *cof1-18 cof1-21* and *cof1-22* (Figure 4.4B).

A)

B)

C)
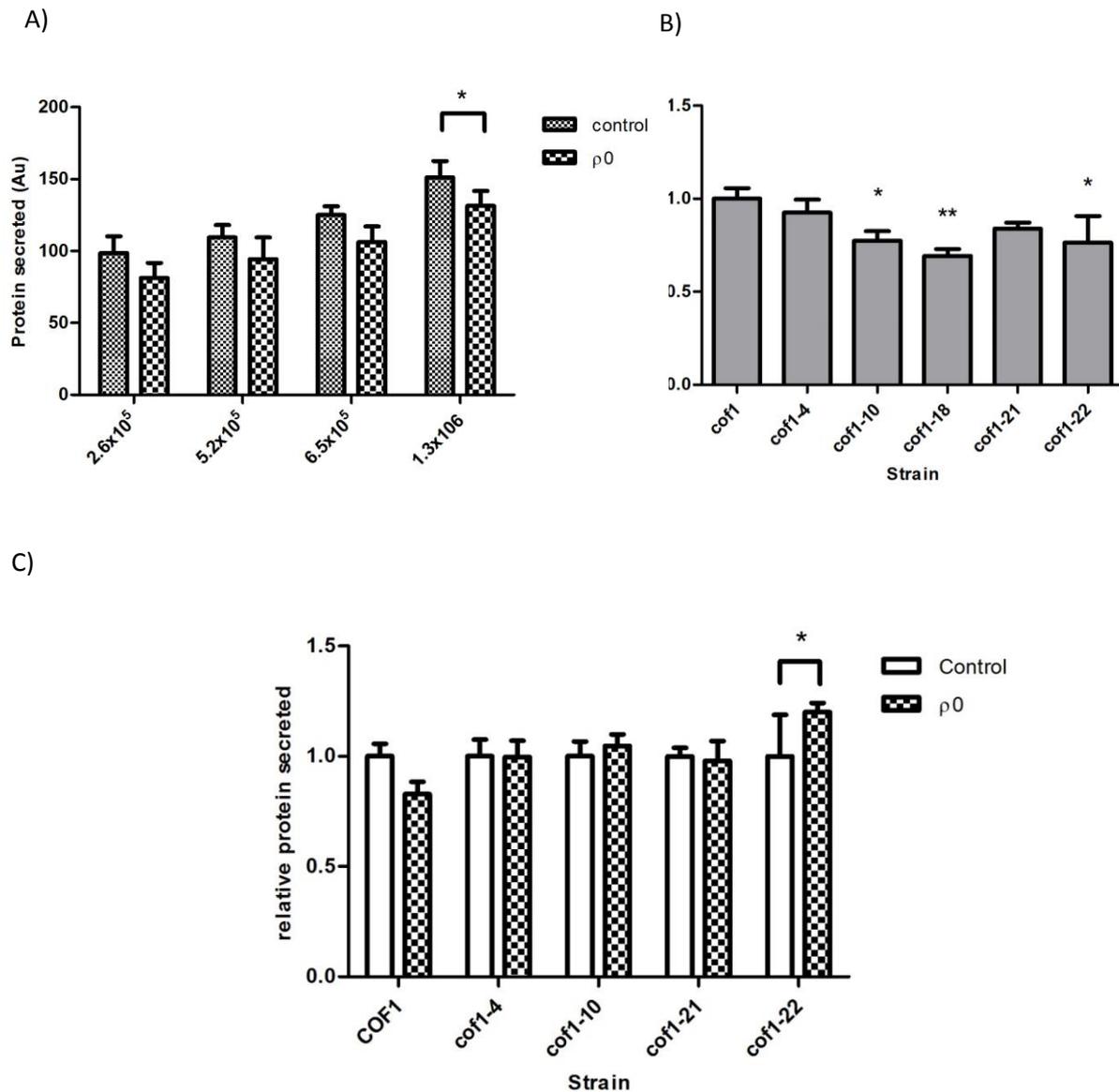
Figure 4.4 Killer toxin secretion in cofilin mutant alleles with mitochondrial DNA removed. A) Average results from quantification of the dilution series of *COF1*. Dilutions were performed in the killer strain with a 5-fold, 2.5- fold and 2-fold dilution (right to left) of the final spot with the cell count $1.3x10^6$. (n=5). B) Quantification of the kill zone from all control strains relative to wild type of the final spot with the cell count $1.3x10^6$. C) Quantification of the kill zone from all control strains relative to petite strains of the final spot with the cell count $1.3x10^6$ .Note there is no data for petite strain for cells expressing *cof1-18*. The kill zone was measured in Image J for quantification. Killer strains were grown overnight in selective media and reinoculated the following morning to a cell OD_{600} of 0.2 and grown to the cell count $1x10^7$. Once spotted on, these plates where left to grow at 30°C for 2-3 days depending on how long the sensitive strain need to grow (n=3). Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test (*P<0.05, **P<0.01, ***P<0.001).

We discovered that with increasing cell number, there was an increase in clearance in both wild type control and petite cells at varied cell counts, yet levels were consistently lower in petites (Figure 4.4A). We observed reduced secretion of the killer toxin in *cof1-10*, *cof1-18* and *cof1-22* when comparing these to the wild type (Figure 4.4B), yet when comparing secretion between the control strains and their petites only *cof1-22* petite had an increase in killer toxin secretion. In conclusion, this data suggests cofilin and mitochondrial function can influence killer toxin secretion.

4.3 Investigating Interactions between Cofilin and TORC1 signalling

Given the role that Cofilin has in connecting signalling mechanisms to mitochondrial function we investigated whether the expression of cofilin mutant alleles led to changes in pathways known to affect mitochondrial biogenesis in yeast. Previous studies have identified that TORC1 signalling is involved in controlling mitochondrial metabolism and biogenesis as well as the control of protein synthesis (Laplante and Sabatini, 2009). We therefore investigated whether the expression of mutant alleles of cofilin that elevated mitochondrial biogenesis also exhibited changes indicative of TOR signalling.

4.3.1 Increased rapamycin resistance in strains expressing cofilin mutant allelles

Alterations in TORC1 signalling can lead to changes in sensitivity to the inhibitory drug rapamycin. Rapamycin inhibits signalling through the TORC1 complex and induces a number of changes, for example its application leads to cell cycle arrest and entry into G0 (Rohde *et al.*, 2001; Smets *et al.*, 2010). FKB12 (a TORC1 binding protein) has been shown to be the target of rapamycin and its presence is required for its actions upon the cell (Michnick *et al.*, 1991; Fingar and Blenis, 2004).

Rapamycin is an immunosuppressive drug that forms a protein-drug complex with FKBP12, interacting with the FRB (FKBP12-rapamycin binding) domain of TOR protein (Shertz *et al.*, 2010). This may block interactions between FRB domain in TOR and other regulatory proteins downstream such as Sch9, or alter the composition of the multiprotein TORC1 preventing function of this essential pathway (Fingar and Blenis, 2004).

We therefore employed resistance to rapamycin treatment as an indicator of TORC1 signalling within strains expressing mutant alleles of cofilin in comparison to their wild type parental strain (Figure 4.5).

Cells expressing mutant cofilin alleles were grown on varying concentrations of rapamycin containing agar plates to test at their sensitivity relative to wild type (*COF1*).
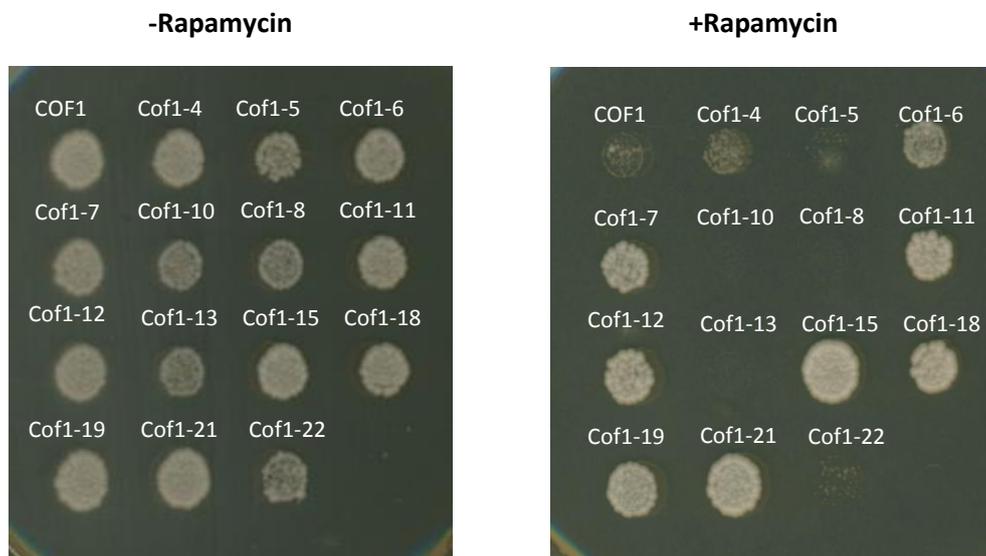
Figure 4.5 Rapamycin resistance of cofilin mutants. Cells were grown overnight in YPD and spotted at a cell OD_{600} of 0.075 and left to grow on YPD agar containing 5ng/ml rapamycin at 30°C for 3 days.

Cells expressing cofilin mutant alleles from Class I showed rapamycin resistance at 5ng/ml in contrast to the wild type cells (Figure 4.5). All mutants expressing cofilin alleles cof1-4, cof1-5, cof1-10, cof1-8, cof1-13 and cof1-22 (all from classes II and III, with exception of cof1-4) were more sensitive to rapamycin when compared to the wild type. This data suggests that mutants from class I, which have elevated levels of mitochondria and respiratory function, may have elevated levels of TORC1 signalling. However it may also be the case that rapamycin is recovered from these cells by the action of drug pumps, or that resistance is mediated by some other mechanism that sequesters the drug or prevents its entry into cells. The increased sensitivity of mutant strains from class II and III, which exhibit mitochondrial dysfunction, may reflect an inability to regulate TORC1 signalling, a reduced capacity to extrude the drug or an increased rate of uptake.

Microarray data from previous work in the Gourlay lab has shown the upregulation of drug pump activity in a *PDR1*-dependent manner in cells expressing allele *cof1-6* (Kotiadis *et al.*, 2012)*.* It is possible that an upregulation of drug pumps may explain the rapamycin resistance results observed in figure 4.5. To test if rapamycin is a substrate for drug pumps, we measured growth while treating the cells with rapamycin alone or in combination with FK506 which inhibits the major ABC transporter

PDR5 in yeast (Egner *et al.*, 1998; Hendrych *et al.*, 2009). Strains expressing cofilin mutant alleles were selected for this experiment which had shown a slight increase (list strains e.g. *cof1-4*) and a significant increase (list strains e.g. *cof1-18*) in rapamycin resistance (Figure 4.6).

A)
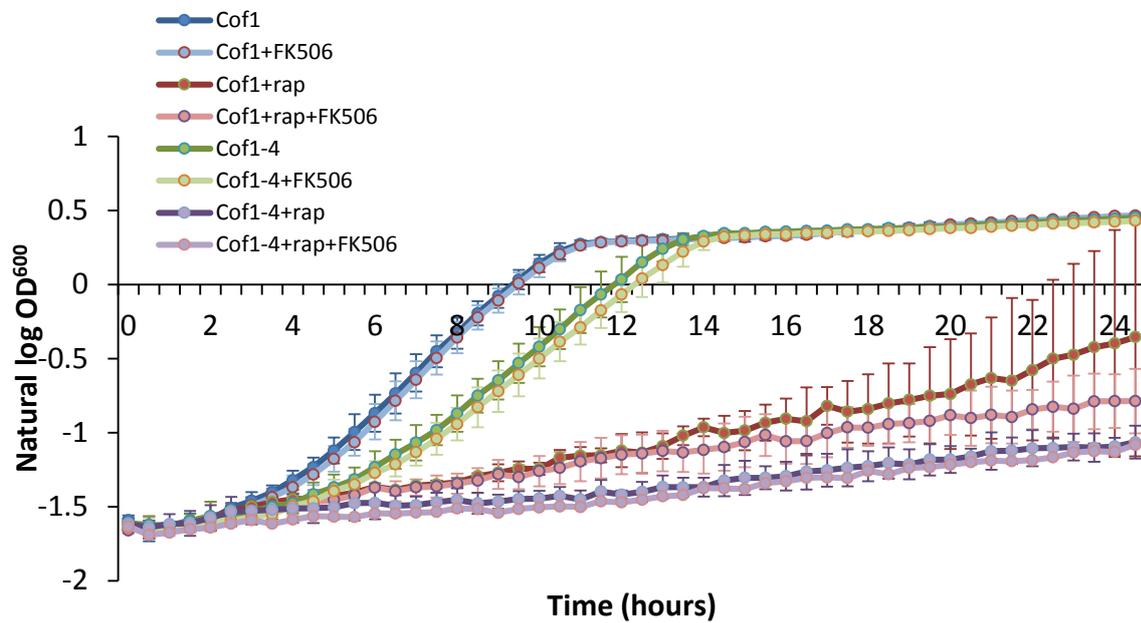
B)
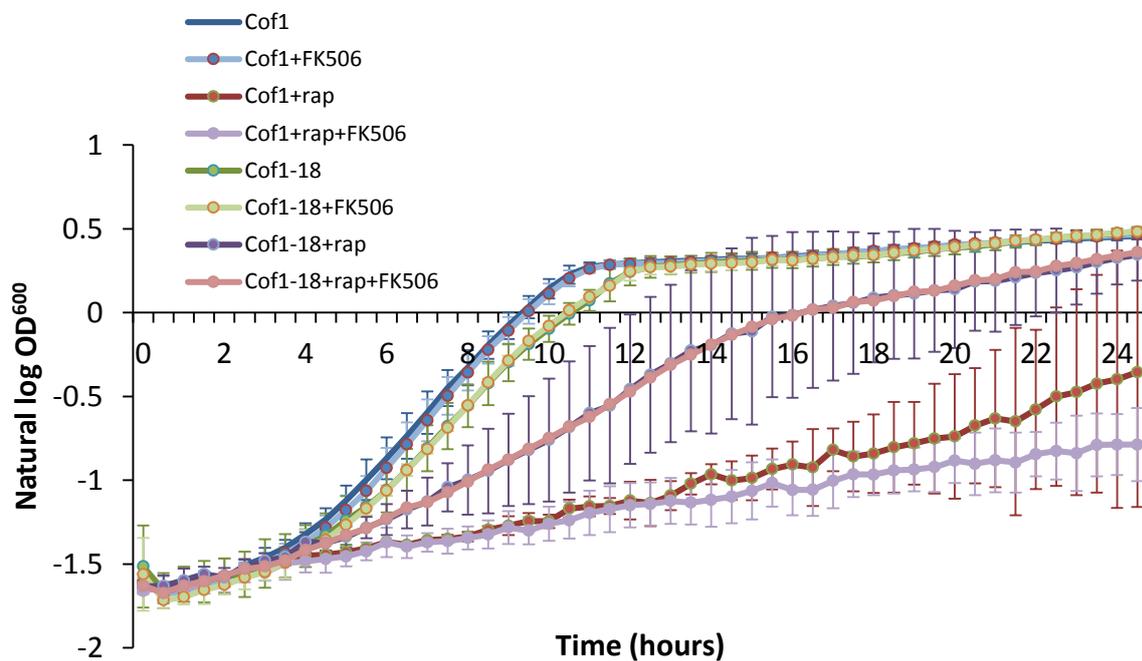
Figure 4.6.1 Drug pump substrate test. A-B) Examples of growth from various cofilin mutant alleles in YPD containing 4ng/ml rapamycin and 1ng/ml FK506. Cells were grown over night before being reinoculated to an OD_{600} of 0.3 with various drugs. A slightly lower concentration of rapamycin was used than in figure 4.5 as cells appeared more sensitive in liquid media. Error bars represent standard deviation.
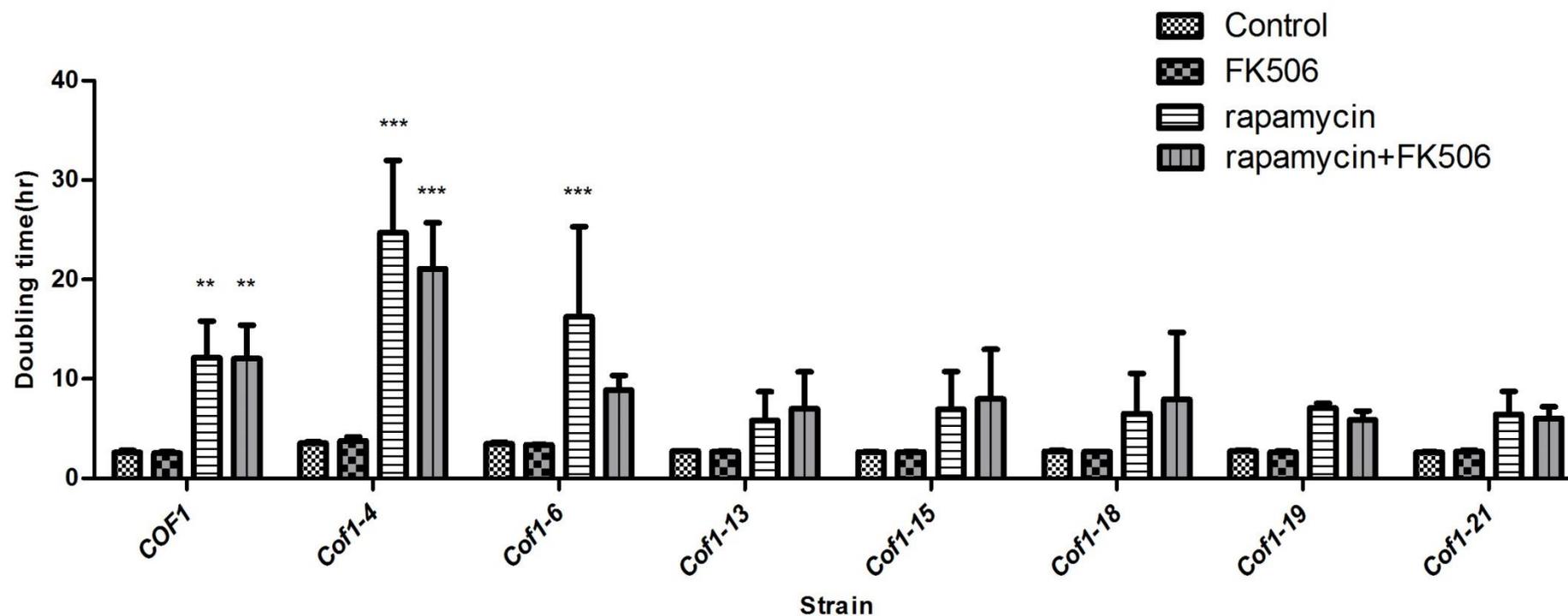
Figure 4.6.2 Drug pump substrate test. A comparison of doubling times with rapamycin and FK506 between mutant alleles. Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test comparing growth of each strain in each drug compared to control growth (*P<0.05, **P<0.01, ***P<0.001). A slightly lower concentration of rapamycin was used than in figure 4.5 as cells appeared more sensitive in liquid media.

Overall, no effect on growth was observed when all strains grown in the presence of FK506 (Figure 4.6.2). It was observed that rapamycin treatment alone and in conjunction with FK506 slowed growth in all strains; yet the wild type, mutant allele *cof1-4* and *cof1-6* had less resistance to rapamycin with a significant increase in doubling time compared to growth without any drug (Figure 4.6.2). The growth curves seen in figure 4.4.1 show the same growth profile of mutant expressing alleles *cof1-13*, *cof1-15*, *cof1-18, cof1-19* and *cof1-21* with the different treatments.

In comparison to all other strains expressing cofilin mutant alleles, cells expressing allele *cof1-4* and *cof1-6* appeared more sensitive to rapamycin in liquid media than on agar media. In contrast to what we had observed on agar, mutant expressing allele *cof1-13* has resistance to rapamycin in liquid media containing rapamycin. Our findings suggest that rapamycin is not a substrate for the major multi-drug pump Pdr5 which is inhibited by FK506 treatment.

As a control to confirm that FK506 treatment was inhibiting the action of the drug pump Pdr5, we incubated cells with itraconazole, a known substrate of ABC transporters and of Pdr5 (Hendrych *et al.*, 2009). Rapamycin resistant cofilin mutants *cof1-6*, *cof1-12* and *cof1-18* from class I were tested for itraconazole sensitivity in the presence or absence of FK506 with respect to wild type (Figure 4.7).
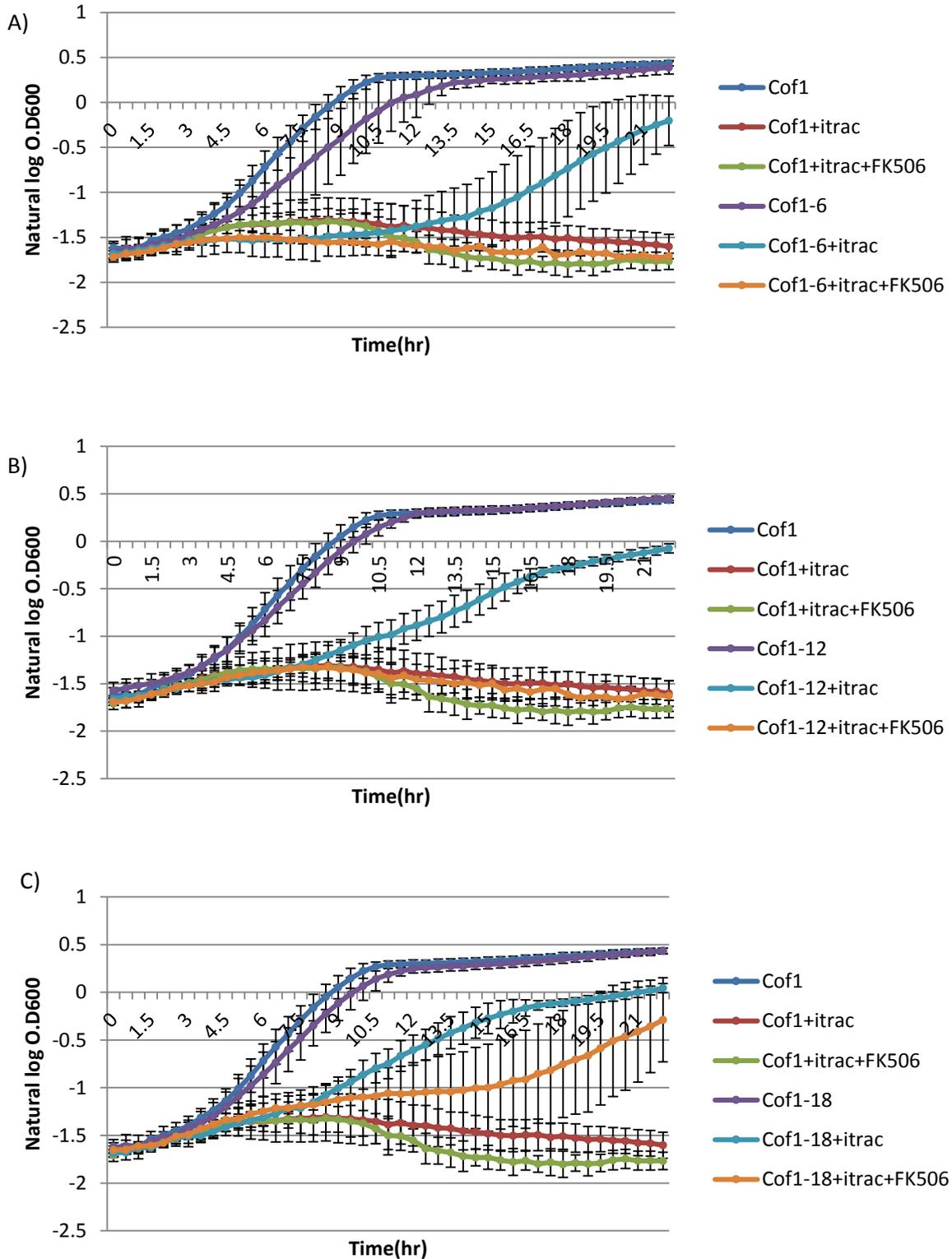
Figure 4.7 Resistance of *cof1-6, cof1-12* and *cof1-18* to itraconazole with FK506. A) Growth of cells expressing the *cof1-6* allele in 6.6µM itraconazole and 1ng/ml FK506. B) Growth of cells expressing the allele *cof1-12* and *cof1-18* (C). Cells were grown overnight before being re-inoculated to an OD_{600} of 0.3 with treatment in selection media. Errors bars represent standard deviation (n=3).

In all strains expressing cofilin mutant alleles we observed resistance to Itraconazole when compared to wild type cells (Figure 4.7). This resistance was either completely abolished, in the case of *cof1-6* or *cof 1-12*, or significantly reduced, in the case of *cof1-18*, in the presence of FK506 (Figure 4.7) This suggests that cells expressing cofilin allele *cof1-18* may have higher upregulation of drug pumps and may need higher concentrations of FK506 to see a loss of resistance to Itraconazole.

For further confirmation that the rapamycin resistance observed in cofilin mutant alleles in class I are not due to the activity of drug pumps, we deleted *PDR1* in *COF1*, *cof1-11*, *cof1-12*, *cof1-15* and *cof1-18* resistant cofilin mutant alleles (all from class I) using a *HIS3* gene disruption cassette. *PDR1* is a transcription factor which controls the transcription of many drug pumps such as PDR5 (Butow and Avadhani, 2004). The effects of *pdr1* deletion in strains expressing cofilin mutant alleles was then tested by growing cells on agar containing rapamycin (Figure 4.8).
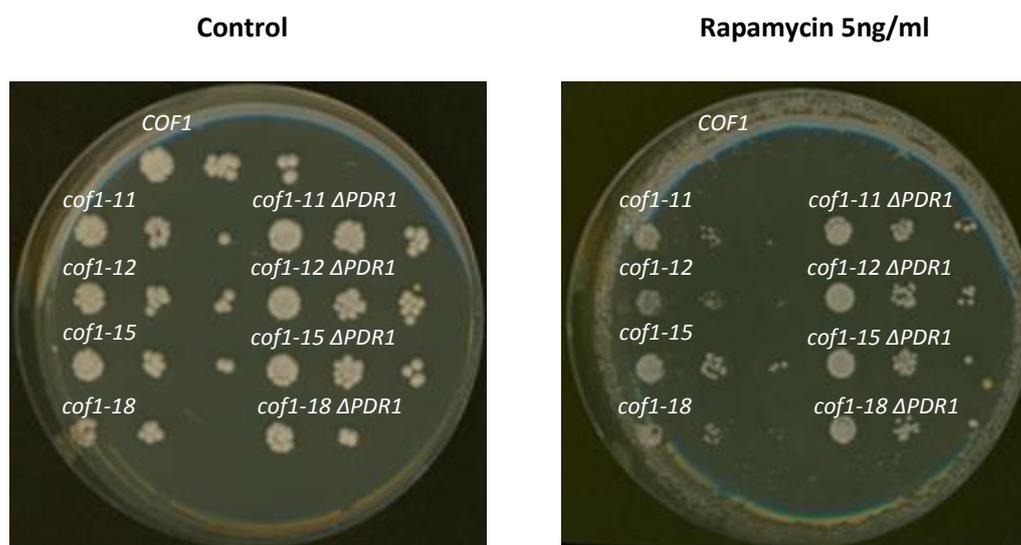
Figure 4.8 Rapamycin resistance test with PDR1 deletion mutants. Cells were grown overnight and spotted at a cell OD_{600} of 0.075 and left to grow on YPD agar containing 5ng/ml rapamycin at 30°C for 2-3 days. A dilution series was done with a 10 fold decrease in concentration.

No growth was observed in wild type (*COF1*) samples spotted onto DO His plates containing 5ng/ml of rapamycin (Figure 4.8). However in all cases, the deletion of *PDR1* in strains expressing cofilin mutant alleles did not lead to a loss rapamycin resistance. On the contrary, when comparing growth of the cofilin mutant strains, the deletion of *PDR1* led to an increase in resistance to rapamycin treatment. This data supports that hypothesis that the rapamycin resistance observed in strains expressing mutant cofilin alleles is not caused by an increase in drug pump gene transcription

controlled by *PDR1*. However this data does not discount the possibility that drug pumps not under the control of *PDR1* are responsible for the rapamycin resistance observed.

To further investigate this we identified the non-essential genes with a Gene Ontology assignment of Drug transporters using the *Saccharomyces cerevisiae* genome database. This list represents a small collection of transcription factors and drug pumps (table 1). We tested whether deletion of each of these genes led to changes in rapamycin or itraconazole sensitivity, to identify which, if any, are needed for resistance. Strains were acquired from the *S. cerevisiae* genome wide knock out collection which are derived from the wild type strain BY4741 (Winzeler *et al.*, 1999).

Table1 List of transcription factors for MDR (multi-drug resistance genes) and drug transporters which are non-essential in yeast. Saccharomyces genome database and UniProt was used to collect this information.

Gene name	Role	Regulation
PDR1	Transcription factor for MDR	PDR10, PDR5,
PDR3	Transcription factor for MDR	PDR10, PDR5
PDR5	drug transporter	
PDR8	Transcription factor for MDR	PDR15, YOR1
PDR10	drug transporter	
PDR11	drug transporter	
PDR12	drug transporter	
PDR15	drug transporter	
PDR18	drug transporter	
YRR1	Transcription factor for MDR	
YOR1	drug transporter	
SNQ2	drug transporter	

| Control | Rapamycin 3.75ng/ml | Itraconazole 6.6µM |
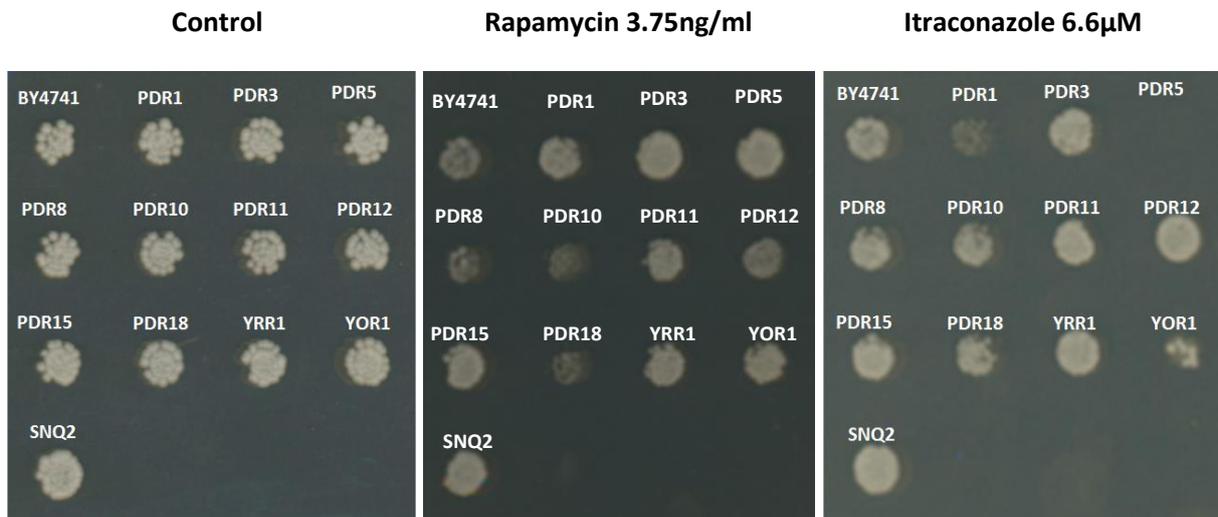
Figure 4.9 Drug pump deletion screen with rapamycin (3.75ng/ml) and 6.6µM Itraconazole. Cells were picked form agar plates and grown overnight in YPD and spotted at a cell OD$_{600}$ of 0.075 and left to grow on YPD agar at 30°C for 3 days.

When compared to wild type, the deletion of multi-drug transporters *PDR10* and *PDR18* (Figure 4.9), as well as the TF *PDR8* led to increased rapamycin sensitivity when compared to the wild type (BY4741). This may indicate that these genes are needed for rapamycin resistance. Interestingly, it was PDR10 which was upregulated in micro array analysis comparing cofilin mutant allele *cof1-6* to the wild type (Kotiadis *et al.*, 2012). As expected Itraconazole treatment caused growth inhibition in strains *Δpdr1*, *Δpdr5* and *Δyor1* (another drug transporter). Our data may suggest that itraconazole and rapamycin are substrates for different drug pumps (Figure 4.9). Further analysis would be required to confirm whether rapamycin may be pumped out by *PDR10* and *PDR18* in response to the activity of which may have TF *PDR8*.

4.3.2 Investigating altered Sch9 signalling with altered cofilin mutant alleles

Although our data suggests that drug pump activity may play a role in resistance to rapamycin, another possibility is that TORC1 kinase activity is elevated in response to mutations in cofilin. To determine whether cofilin mutant strains exhibit changes in TORC1 signalling, we made use of an assay which assesses the phosphorylation status of Sch9, a major downstream target of TORC1 signalling. Sch9 phosporylation affects a number of processes in the cell, including growth and ribosomal biogenesis (Hands *et al.*, 2009). Due to the large size of this protein (~100kDa) and the presence of multiple

phosphorylation sites, NTCB (2-nitro-5-thiocyanatobenzoic acid) was used to induce chemical fragmentation as previously described (Urban *et al.*, 2007). Treatment with NTCB selectively cyanylates cysteine thiol and under alkaline conditions cleaves on the N-terminal side of the residue, which in turn leads to protein fragmentation(Wu *et al.*, 1996). Using this method, fragments of Sch9 are generated and changes in phosphorylation can be detected by SDS-PAGE. C-terminally HA-tagged Sch9 was detected by western blotting using an anti-HA antibody following fragmentation as described (Urban *et al.*, 2007). Sch9 phosphorylation was tested using this method in strains expressing the alleles *cof1-11* and *cof1-18,* which both displayed elevated rapamycin resistance and *cof1-5* (only 4.10B) which was not resistant, during both log and stationary phase of growth (Figure 4.10).

A)
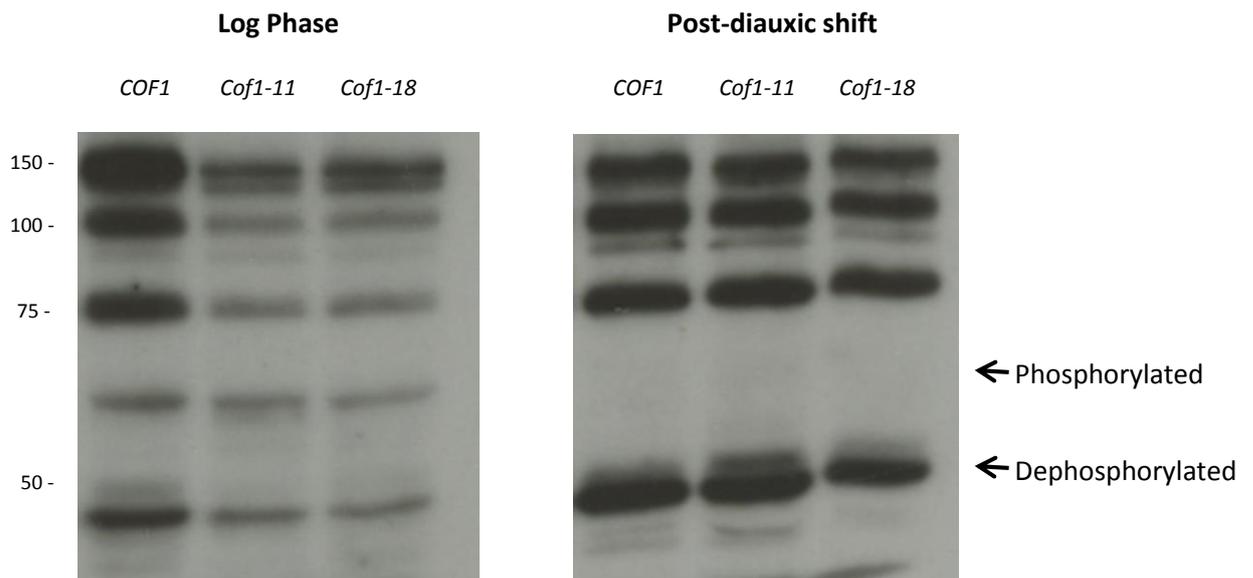
B)
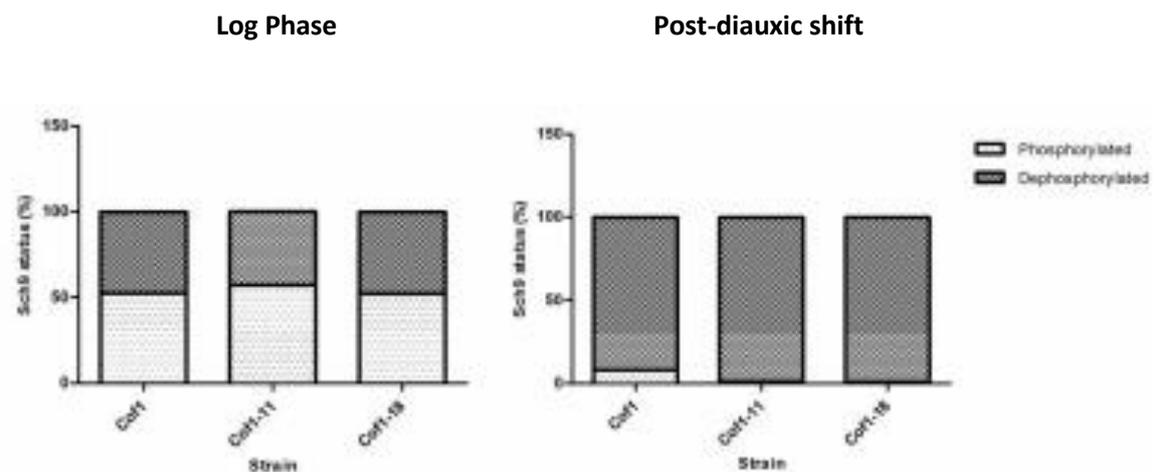
Figure 4.10a Chemical fragmentation analysis of Sch9 phosphorylation during log phase and post diauxic shift. A) blots showing phosphorylated and dephosphorylated C-terminal sch9. B) Percentage of Sch9 phosphorylated and dephosphorylated. Cells were grown in selection media overnight and re-inoculated to OD_{600} 0.3 and left to grow into log and post-diauxic shift. Sch9 was detected using an anti-HA antibody.

A)
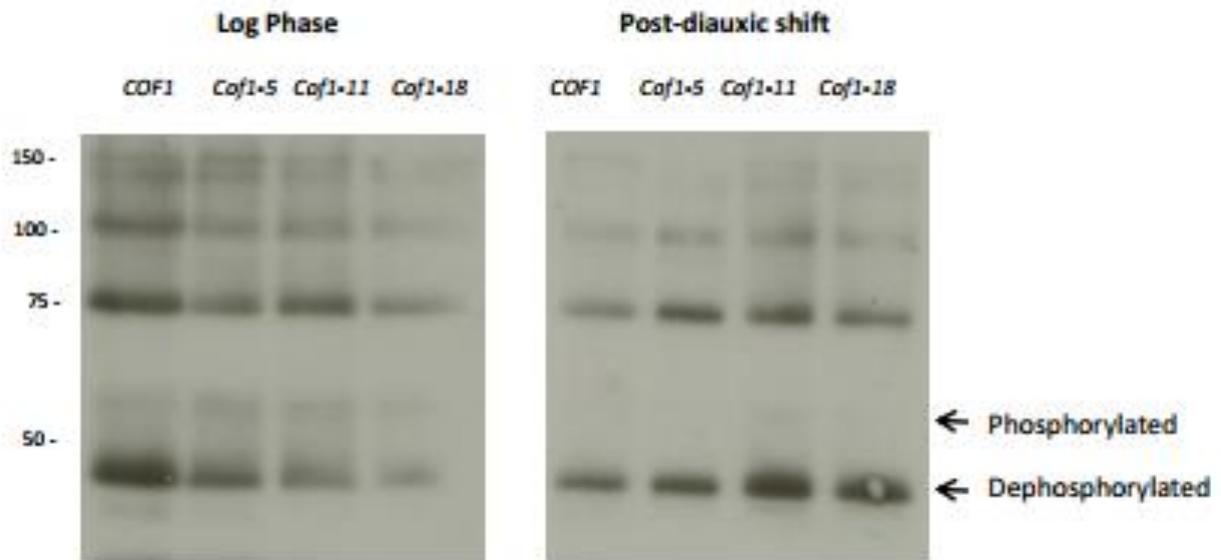
B)
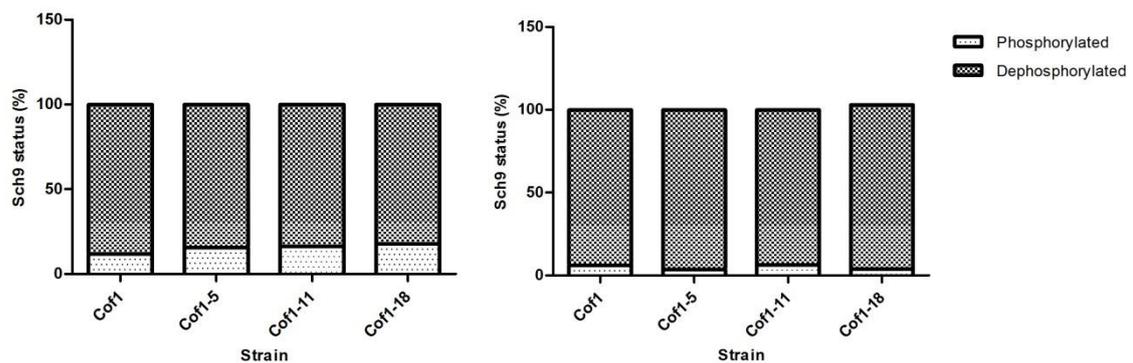
Figure 4.10b Repeat of chemical fragmentation analysis of Sch9 phosphorylation during log phase and post diauxic shift. A) blots showing phosphorylated and dephosphorylated C-terminal sch9. B) Percentage of Sch9 phosphorylated and dephosphorylated. Cells were grown in selection media overnight and re-inoculated to OD_{600} 0.3 and left to grow into log and post-diauxic shift. Sch9 was detected using an anti-HA antibody.

When comparing the levels of Sch9, we observed higher phosphorylation during log phase compared to post diauxic shift phase across all strains from both repeats (Figure 4.10A/B). This is expected as TORC1 is active during rapid growth which should lead to Sch9 phosphorylation. Sch9 was not detectable in all strains tested as cells moved into post-diauxic shift suggesting a reduction in TORC1 activity. This data suggested that TORC1 activity is comparable during log and stationary phase cells in wild type and cofilin mutant strains, however there were no significant differences between strains with varied rapamycin resistance. When comparing the repeats on separate days, (Figure 4.10A/B)

higher levels of phosphorylated sch9 where detected in Figure 4.10A. However, this does not alter our overall conclusion that there are similar levels of Sch9 active between the wild type and mutants.

4.4 Roles of Cofilin in regulating MAPK signalling

MAPK signalling is important for not only mating in yeast, but for environmental sensing and response to a range of stimuli (Brückner *et al.*, 2011). There are four clearly defined MAPK pathways involved controlling mating, filamentous growth, high osmolarity and cell wall integrity. The kinase Hog1 (High-Osmolarity-Glycerol) is needed for survival under hyperosmotic conditions and can be activated via two pathways (Molina *et al.*, 2010). The first is by phosphorylation of Pbs2 under iso-osmotic conditions and the second is via Ste11; activation of Hog1 causes it's translocation from the cytoplasm to the nucleus where it affects the expression of genes in response to hyperosmotic shock (Chen and Thorner, 2007). Previous work has indicated that MAPK signalling can be affected by mutations in cofilin (C. Gourlay, unpublished work). We therefore sought to determine the extent to which cofilin interacts with MAPK signalling pathways in yeast.

4.4.1 Cofilin and activation of the p38 stress linked MAPK HOG1

Yeast adapt rapidly to adverse environmental conditions. Changes to the environment act as cues, signalling to specific MAPK pathways (Bardwell, 2006). When there is an increase in dissolved solute concentration of the external medium in comparison to the internal osmolarity of the cell it causes a drop in turgor pressure. To deal with this, the cell's internal osmolyte concentration is altered by increasing their synthesis of glycerol. This mechanism is activated by the High Osmolarity Glycerol (HOG) MAPK pathway which requires activation of Hog1, which is a functional ortholog p38 family in mammalian cells (Westfall *et al.*, 2004; Rodríguez-Peña *et al.*, 2010). Activated Hog1 MAPK induces cellular osmo-adaptive responses such as accumulation of the osmolyte glycerol, inhibition of protein synthesis and temporary arrest of the cell cycle in G1 phase. There are two branches of the HOG pathway. The SLN1 branch activates Ssk2 and Ssk22 compared to the SHO1 branch that activates Ste11 (see figure 1.5). Both branches activate Pbs2 (MAPKK) and subsequently activate Hog1 (MAPK) (Molina *et al.*, 2010; Saito, 2010). Hog1 influences the expression of a wide variety of TFs; those grouped together are sequence related. These include Hot1 and Msn1, Msn2 and 4, Sko1, as well as Smp1 (Alepuz *et al.*, 2001; Capaldi *et al.*, 2008).

HOG1 is the yeast homologue of the human stress activated kinase p38MAPK. Previous studies have determined that an antibody that detects phosphorylation of p38MAPK in human cells is also able to report phosphorylation of Hog1 in yeast (Widmann and Gibson, 1999; Martín *et al.*, 2005). We therefore examined the total levels of Hog1 and its phosphorylation in wild type and cofilin mutant strains *COF1*, *cof1-5*, *cof1-8*, *cof1-12*, *cof1-19* and *cof1-21* as well as *Δhog1* in response to salt stress (Figure 4.11). Cells lacking the *HOG1* gene were used as a negative control in these experiments.
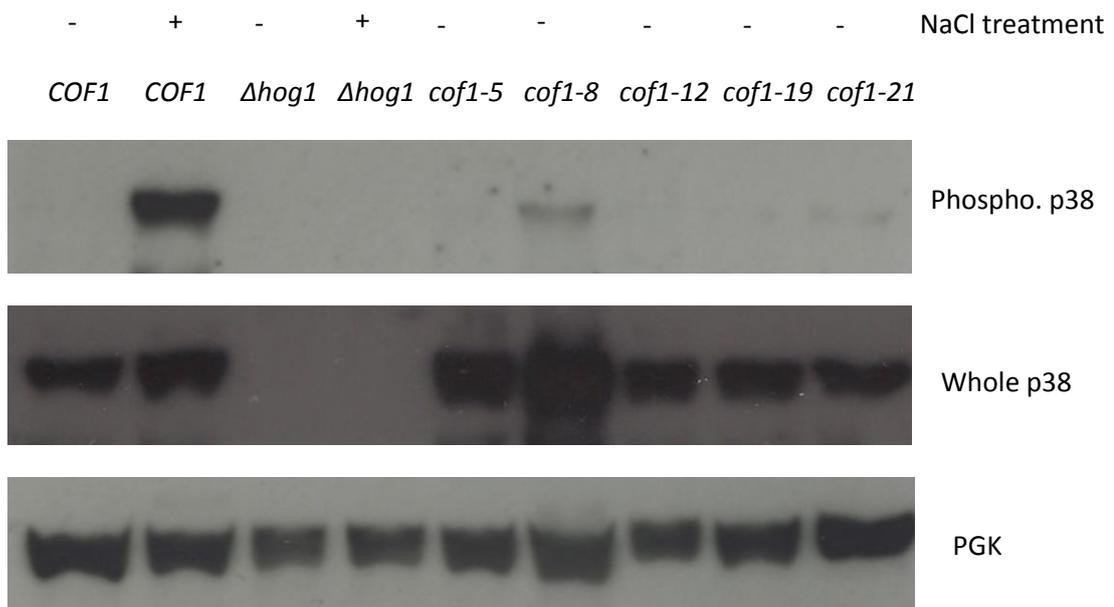
Figure 4.11 Expression of phosphorylated and whole Hog1 in log phase with salt stress. Western blot showing Hog1 phosphoylation and total Hog1 expression levels. PGK was used as loading control. Cells were grown in selection media overnight and reinoculated to at OD$_{600}$ of 0.3 and left to grow. Strains indicated were treated with 162mM NaCl for 5 minutes at room temperature before protein was extracted using the yeast whole cell quantitative method.

As expected levels of Hog1 phosphorylation were undetectable using western blotting in wild type cells but rose dramatically upon salt stress (Figure 4.11). Strains expressing cofilin mutant allele's *cof1-8*, *cof1-19* and *cof1-21* appeared to display low levels of Hog1 phosphorylation under normal growth conditions suggesting a degree of aberrant signalling. Further work is required to assess the significance of this finding.

4.4.2 Cofilin and activation of the mating /pseudohyphal MAPK pathway

Ste12, the MAPK transcription factor, activates genes in the mating and filamentation pathway which is activated in response to nutrient deprivation(Brückner *et al.*, 2011). For these responses to occur within the cell, Ste12 regulates a different set of genes by binding to specific promoter elements. For mating response genes it binds as a homodimer to the DNA motif PRE (pheromone-regulated genes). However, Ste12 binds as a heterodimer with Tec1 to induce genes for filamentous growth, known to contain FRE (filamentous response element) sequence(Wong Sak Hoi and Dumas, 2010). Previous data has suggested that Ste12 activity is upregulated in a strain expressing the allele *cof1-6* as a potential candidate for upregulating some of the identified genes such as TEC1 and FUS3 (Kotiadis *et al.*, 2012). To determine the extent to which cofilin surface charge alterations can influence Ste12 activity, we employed a Ste12 activity β-Galactosidase assay using a vector containing the FRE promoter with the *lacZ* reporter gene to measure the activity of Ste12 (Figure 4.12).

A)
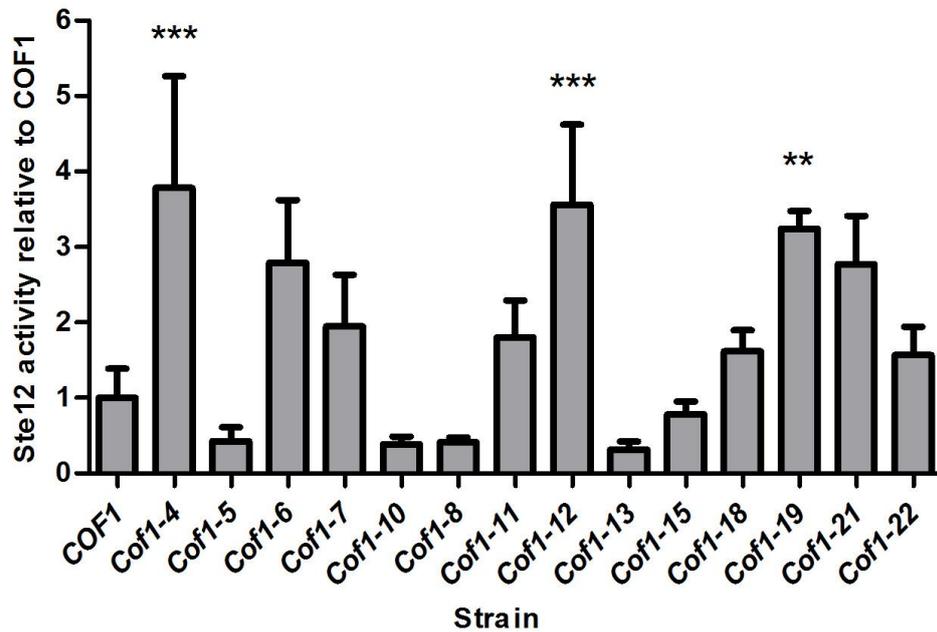
B)

Figure 4.12 Ste12 activity in cofilin mutants strains. Cells were grown overnight in DO URA and re-inoculated to an OD_{600} of 0.3 in minimal media before being left to grow overnight to grow to an O.D of 5 before treated with Z buffer and ONPG to start the reaction. Cells were left to incubate until sufficient colour change was seen and the reaction stopped and its absorbance measured. A) Shows miller units read in β-galactosidase assay B) resistance to rapamycin in strains expressing cofilin mutant alleles (from figure 4.5). Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test (*P<0.05, **P<0.01, ***P<0.001).

Our results show that cofilin mutants in class I exhibited elevated levels of Ste12 activity when compared to the wild type (*COF1*) (Figure 4.12A). In particular, the mutants expressing alleles *cof1-4, cof1-6, cof1-12, cof1-19* and *cof1-21* displayed significant increases in Ste12 activity compared to the wild type (all from class I); in comparison, cells expressing mutant allele *cof1-15* was the only mutant allele from this class to present very low levels. Reduced levels of Ste12 activity were detected in all mutant alleles from class II and III compared to wild type (except expression of allele *cof1-22*). A broad

correlation was therefore observed with high Ste12 activity and those that exhibited resistance to rapamycin (Figure 4.5B).

4.4.3 Investigating whether STE12 activity is linked to rapamycin resistance

To investigate whether changes in Ste12 activity were important in mediating resistance to rapamycin we deleted the gene *STE12* in strains expressing cofilin mutant alleles (Figure 4.12). This method was achieved using a gene disruption cassette which replaced *STE12* by homologous recombination with a HIS3 marker. These cofilin mutant *ste12* deletion strains were spotted onto agar containing rapamycin to test for any changes to resistance (Figure 4.13).
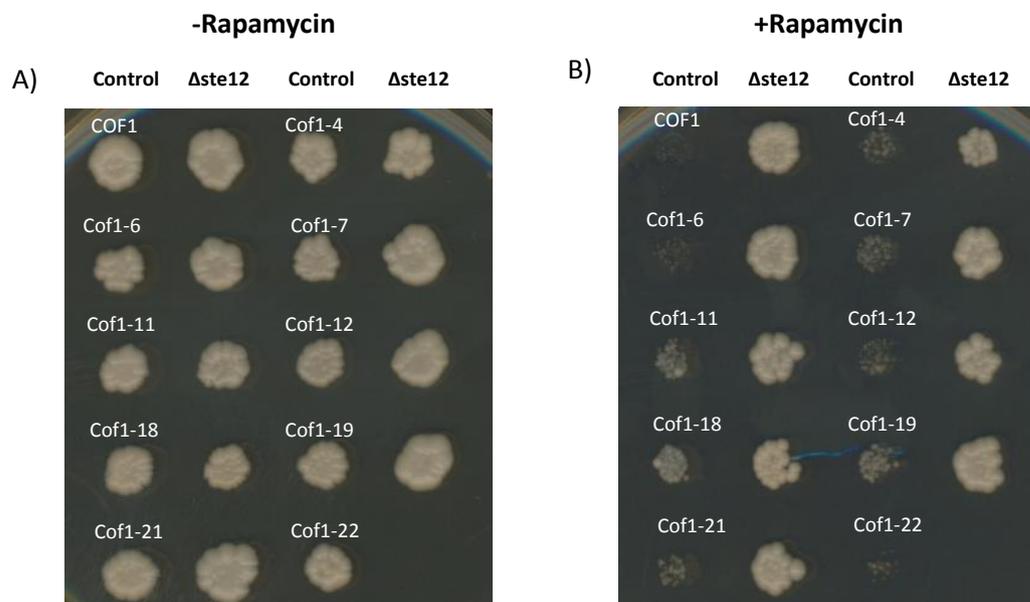
Figure 4.13 STE12 was deleted in cells expressing cofilin mutant alleles. A) Control agar plate B) shows agar containing 6.25ng/ml rapamycin. Cells were grown overnight and spotted at a cell OD_{600} of 0.075 and left to grow on YPD agar containing rapamycin at 30°C for 2-3 days.

Our data demonstrates that in all strains tested the deletion of *STE12* led to a dramatic increase in resistance to rapamycin (Figure 4.13). This suggests that the regulation of STE12 activity is important in mediating resistance to rapamycin, but that its elevation in strains expressing mutant cofilin alleles may not be a contributing factor.

4.4.4 Effect of *STE12* deletion on growth

We considered that changes in rapamycin resistance observed when deleting *STE12* may be may be linked to growth rate. As growth rate changes can sometimes be unclear on solid agar spotting assays we compared the growth of the wild type and selected mutant strains expressing cofilin alleles containing the GLuc expression plasmid grown in liquid growth. Some of the cofilin mutant expressing alleles with *Δste12*, *cof1-4, cof1-18, cof1-21* and *cof1-22* were selected (from all three classes) for growth analysis in comparison to wild type (Figure 4.14).

A)

B)
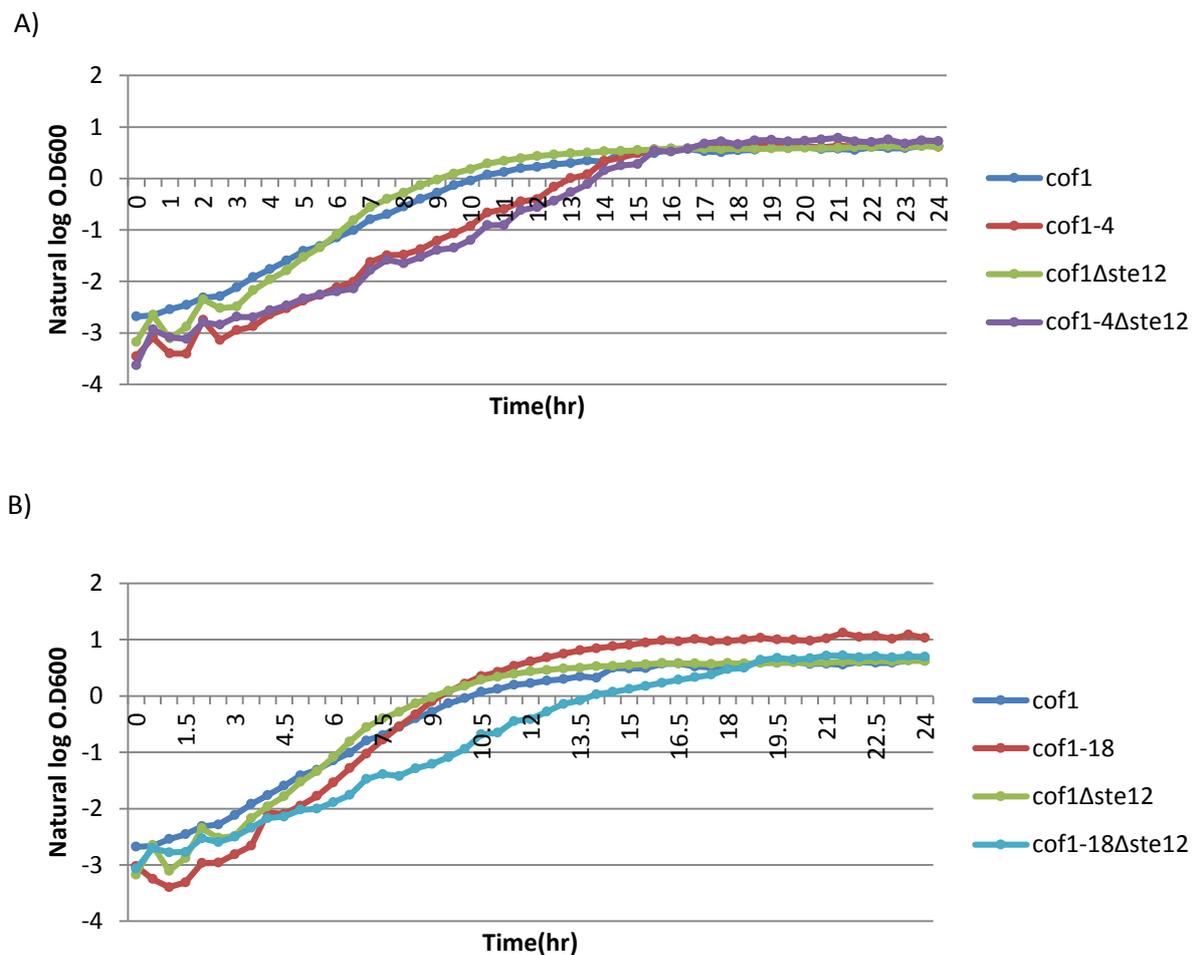
Figure 4.14.1 Growth of cofilin mutants with *STE12* deleted. Logged growth curves of cofilin mutant expressing A) alleles *cof1-4* and B) allele *cof1-18*. Cells were grown overnight before re-inoculated to an OD_{600} of 0.3 in selection media (n=3). Errors bars represent standard deviation.

A)

B)
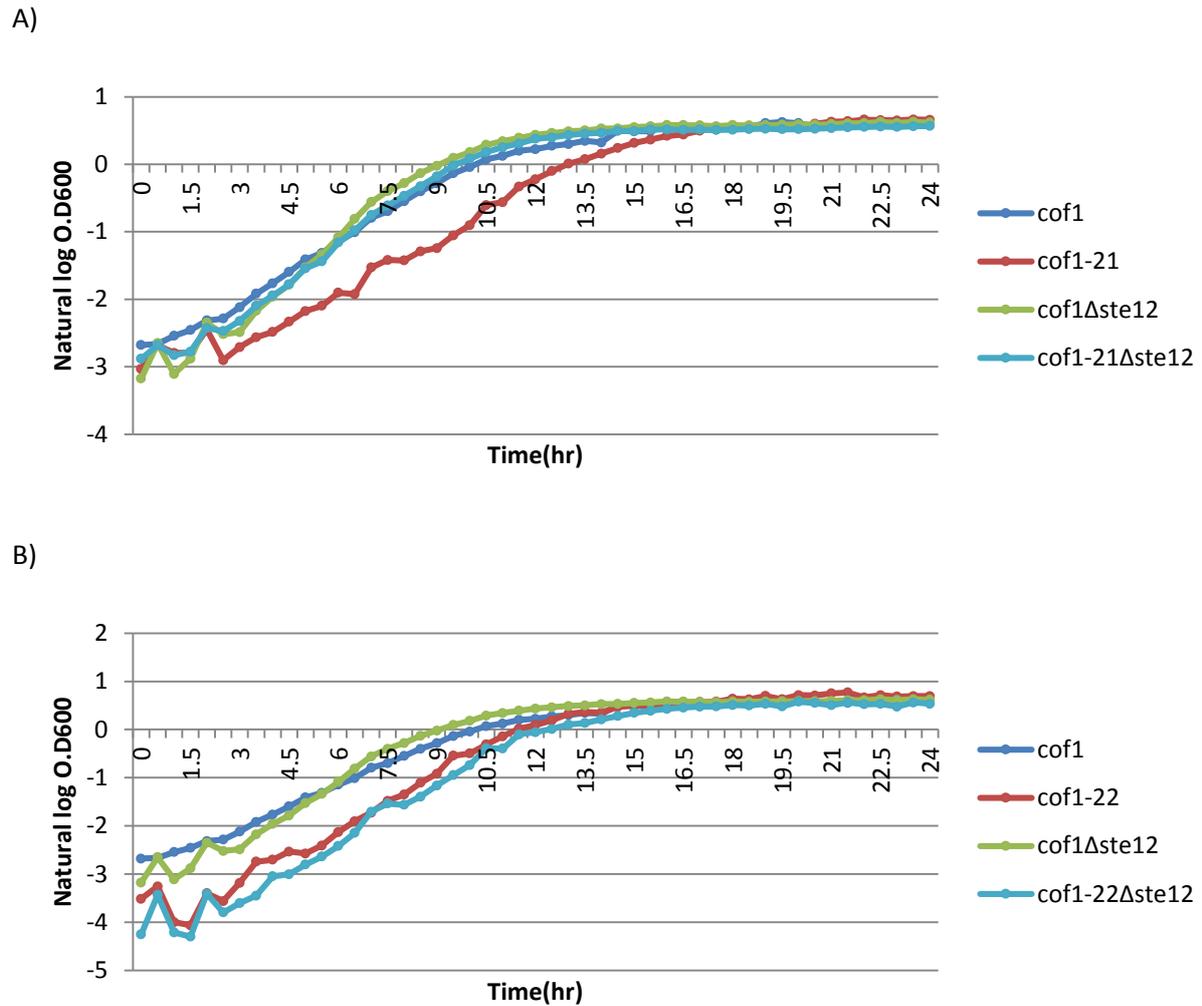
Figure 4.14.2 Growth of cofilin mutants with *STE12* deleted. Logged growth curves of cofilin mutant expressing A) alleles *cof1-21* and B) allele *cof1-22*. Cells were grown overnight before re-inoculated to an OD$_{600}$ of 0.3 in selection media (n=3). Errors bars represent standard deviation.

A)

B)

C)
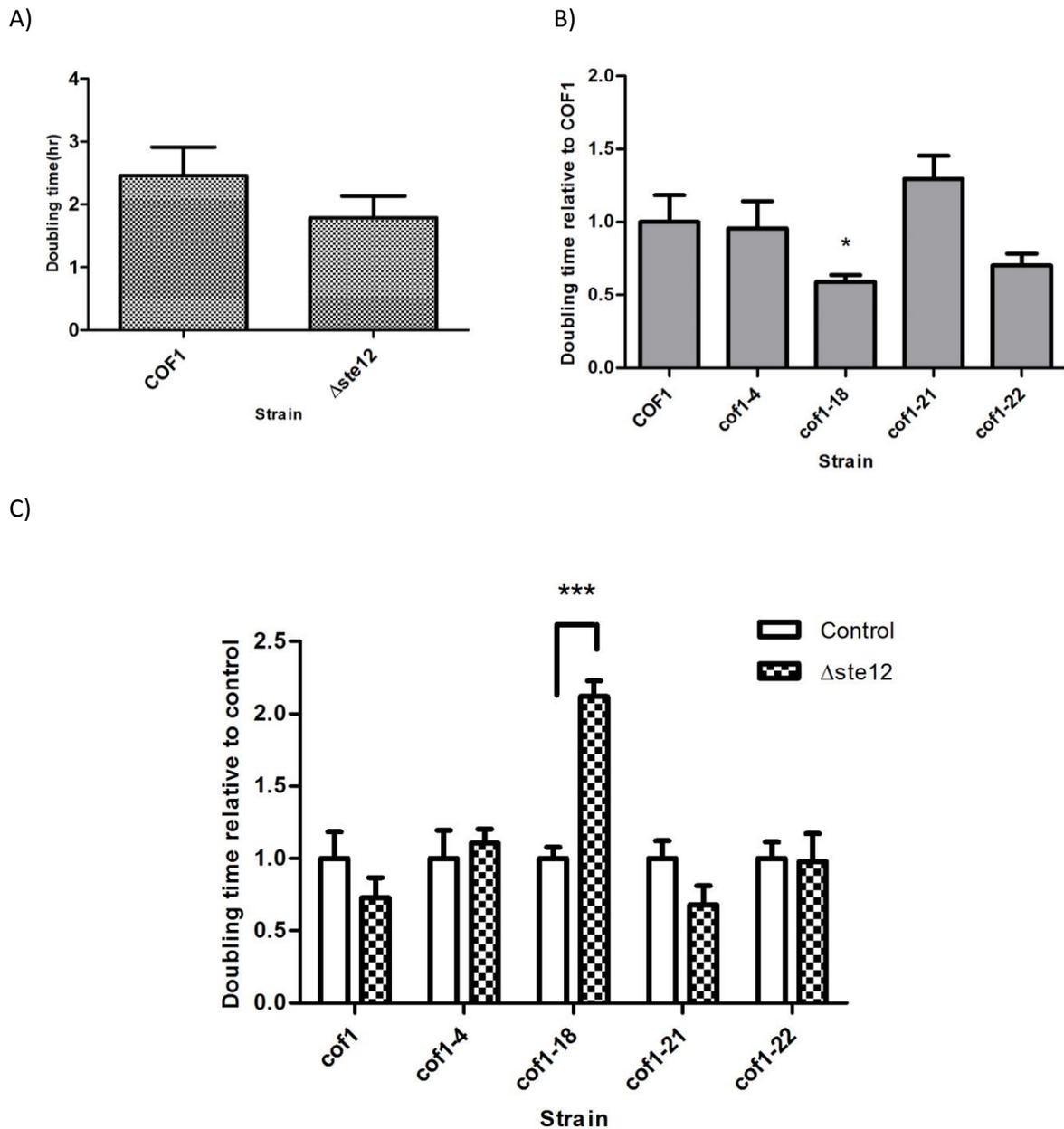
Figure 4.14.3 Quantified growth of GLuc-transformed cofilin mutants with *STE12* deleted. A) Calculated doubling times of the wild type with *Δste12*. B) Doubling time of each cofilin mutant allele relative to the wild type. C) Doubling time of strains with *Δste12* relative to their control. Cells containing the GLuc expression plasmid were grown overnight before re-inoculated to an OD_{600} of 0.3 in selection media (n=3). Errors bars represent standard deviation. Statistical analysis was performed using 2 way ANOVA test (*P<0.05, **P<0.01, ***P<0.001).

When comparing the doubling time of the wild type with and without the *STE12 gene*, we did not observe a significant change in growth rate (Figure 4.14.3A). We identified changes to growth of cells expressing the mutant allele *cof1-18* (Figure 4.14.3B) where there was a decrease in doubling time (1.44hrs compared to the wild type at 2.4hrs). However in cofilin mutant allele *cof1-18 Δste12* cells, the doubling time was further increased to levels similar to the wild type (Figure 4.14.3C). In comparison, deletion of *STE12* had no significant influence on the growth of strains expressing alleles *cof1-4, cof1-21*, and *cof1-22*. This data suggests that the cofilin mutant allele *cof1-18* causes changes to growth through Ste12 signalling, as removing STE12 returns the mutant's growth roughly to the wild type doubling time.

4.4.5 Effects on GLuc expression with MAPK alterations

Our data demonstrates an interaction between cofilin function and the regulation of STE12-MAPK activity. As we have established that cofilin plays a role in the regulation of rP production and secretion, we wished to establish whether STE12 activity was involved. Protein was extracted from cells expressing cofilin mutant allele's *cof1-18* and *cof1-21* which exhibited high Ste12 activity and increased rapamycin resistance, and from the same strains in which *STE12* had been deleted. GLuc expression of these strains were analysed by western blotting (Figure 4.15).
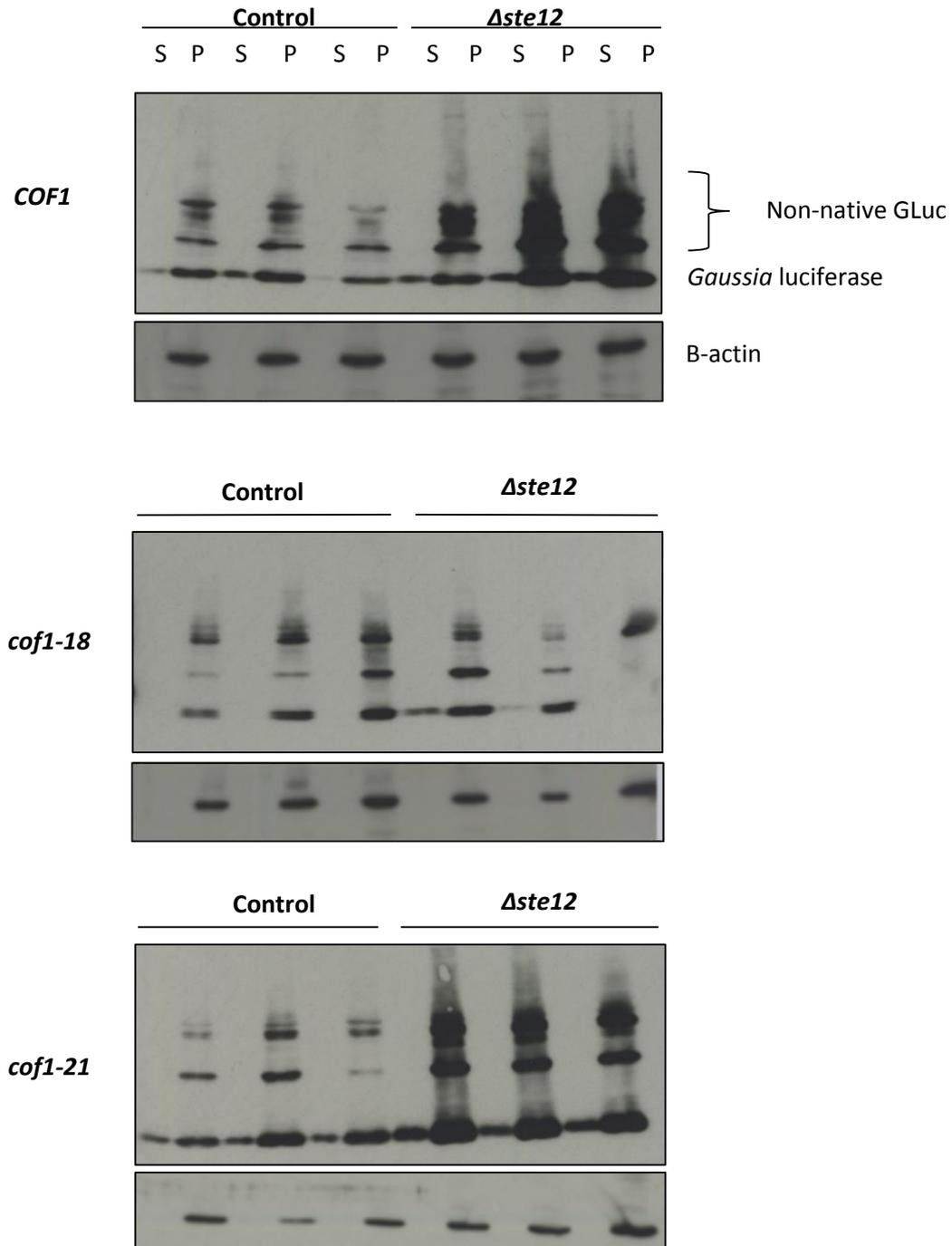
Figure 4.15.1 Expression of GLuc in *STE12* deletions in cofilin strains. Blots of selected cofilin mutant alleles showing GLuc expression. Expression detected in supernatant (s) and pellet (p). Cells were grown overnight in selective media and re-inoculated in standard YPD at OD_{600} 0.1 and grown to the cell count 1×10^7. Protein was extracted from the pellet using the yeast whole cell quantitative method and supernatant treated with 2x sample buffer (n=3). Actin was detected in these strains and used to normalise data.
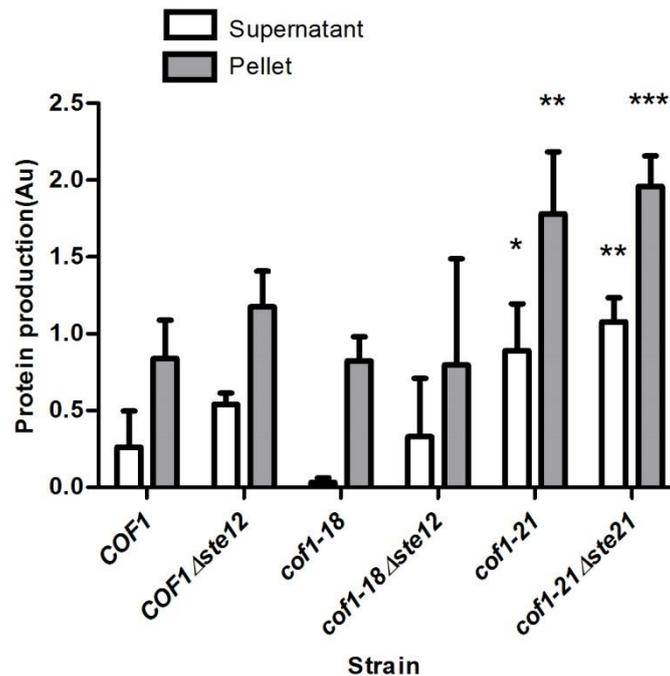
Figure 4.15.2 Quantified expression of GLuc in ste12 deletions in cofilin strains. Cells were grown overnight in selective media and re-inoculated in standard YPD at OD_{600} 0.1 and grown to the cell count $1x10^7$. Protein was extracted from the pellet using the yeast whole cell quantitative method and supernatant treated with 2x sample buffer (n=3). Actin was detected in these strains and used to normalise data. Quantification of these blots using the free software Image J. Errors bars represents standard deviation. Statistical analysis was performed using an ANOVA test comparing GLuc detected in the supernatant between the wild type and each strain as well as the pellet (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

Deleting *STE12* in wild type caused no significant change in the levels of GLuc detected (figure 4.15.2), as was also the case for mutant allele *cof1-18*. Although there was an increase in mutant allele *cof1-21Δste12* compared to the wild type, this was not statistically different to the mutant allele still containing the *STE12* gene.

We were interested in any changes caused to the incorrectly processed forms of GLuc also shown in 4.15.1, where other heavier bands appear in western blot. Figure 4.16 shows quantified GLuc expression from all products appearing in the pellet to test if *Δste12* affects the incorrectly processed forms.

A)

B)

C)

D)
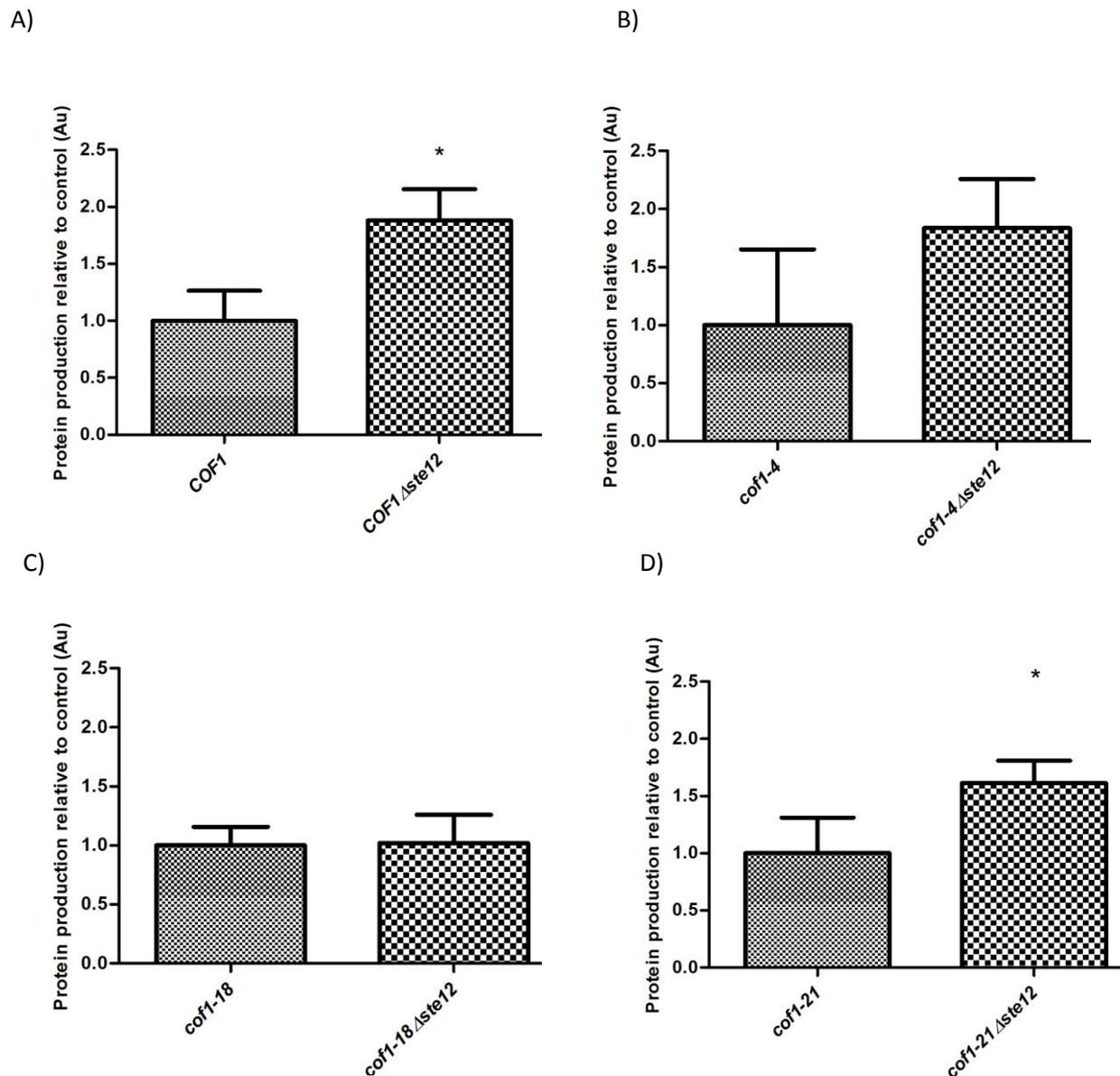
Figure 4.16 Quantified expression of all forms of GLuc in STE12 deletions relative to their control in cofilin strains found in the pellet. A) Quantified GLuc expression in the wild type COF1. B) Quantified GLuc expression in mutant allele *cof1-4*. C) Quantified GLuc expression in mutant allele *cof1-18* D) Quantified GLuc expression in mutant allele *cof1-21*. Errors bars represent standard deviation. Statistical analysis was performed using the unpaired t-test comparing change in protein production by *Δste12* relative to the control (n=3). (*P<0.05, **P<0.01, ***P<0.001).

Δste12 caused a significant increase when quantifying all forms of GLuc (all bands in pellet sample) in the wild type with a very similar change in mutant allele cof1-4, roughly doubling GLuc detected (figure 4.16A/B). The native form of GLuc in *cof1-4* was not shown in figure 4.15.1 due to problems with the transfer creating larger error bars making it difficult to quantify; although this may suggest *Δste12* increases incorrectly processed forms of GLuc production. *Cof1-21* had also shown a significant

increase in GLuc production in the pellet; in contrast *cof1-18* had shown no change (Figure 4.16C/D). There is strong evidence that Ste12 signalling is involved in recombinant protein production independent of the cofilin protein (Figure 4.16).

To determine whether the loss of STE12 also led to changes in secretion in all secreted protein, we again made use of the killer toxin assay (Figure 4.17). Secretion of killer toxin was assessed in cofilin mutant alleles *cof1-4*, *cof1-18*, *cof1-12* and *cof1-22* during log phase, the same strains used to test GLuc expression.
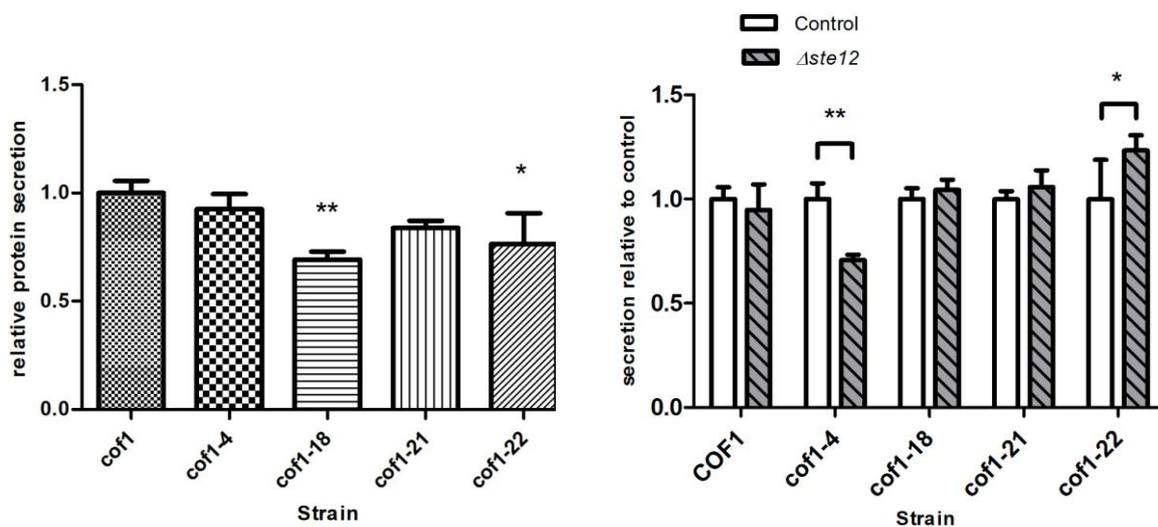
Figure 4.17 The effects of STE12 deletion upon secretion of killer toxin in cofilin mutant strains. A) Killer toxin secretion of cofilin mutant alleles relative to the wild type. B) Killer toxin secretion of each control strain relative to *Δste12*. Killer strains were grown overnight in selective media and re-inoculated the following morning to a cell OD_{600} of 0.2 and grown to the cell count $1x10^7$.. Once spotted on, these plates where left to grow at 30°C for 2-3 days (n=3). The kill zone diameter was measured in Image J for quantification. Quantification of the kill zone of all strains with the cell count $1.3x10^6$ Errors bars represents standard deviation. Statistical analysis was performed using an ANOVA test (***P<0.001).

We observed a loss in killer toxin secretion in cofilin mutant allele's *cof1-18* and *cof1-22* (Figure 4.17A) and when comparing secretion of *Δste12* relative to their control, we found mutant allele *cof1-4* had a significant loss in killer toxin secretion and *cof1-22* had an increase in killer toxin secretion (Figure 4.17B). Overall these results are in contrast to my findings with regards to GLuc production.

4.5 Discussion

In this chapter we investigated interactions between cofilin and the control of cell signalling processes that are important for environmental sensing, growth and stress response. Using a library of mutant alleles in which surface charged residues were mutated to alanine, previous work in the Gourlay lab had identified alterations to mitochondrial function placing these alleles into three set groups. These were tested in an array of assays to investigate how these changes may affect rP production.

4.5.1 Mitochondrial function and its importance in recombinant protein production

It is possible that enhanced mitochondrial function could have effects on protein processing in the secretory pathway due to the relationship between the ER and mitochondria as discussed by Vannuvel et al, (2013). This group discussed how these two organelles are linked by the ERMES junction, where calcium is exchanged, activating apoptosis triggered by ER stress. ER stress also modifies the morphology and bioenergetics of mitochondria as early ER stress was found to be more physically connected to the mitochondria, to favour calcium exchange. This change in morphology promotes ATP production, oxygen consumption as well as an increase in mitochondrial membrane potential (Bravo *et al.*, 2011). Therefore, mitochondrial function is necessary for dealing with ER stress; enhanced mitochondrial function may help the cell to be better adapted, having a positive impact on rP production. Other evidence linking this cross talk to rP production suggested that ER stress and mitochondrial dysfunction may cause metabolic disorders such as Type II diabetes, due to altered insulin secretion (Lim *et al.*, 2009).

As previous work from the Gourlay lab had shown that mutant cofilin allele's exhibited altered mitochondrial function (Kotiadis *et al.*, 2012), a GLuc expression construct was introduced into cofilin mutant strains to determine whether such changes would lead to altered rP expression or secretion. We discovered that the mutation of cofilin led to variation in GLuc protein production and secretion, with the highest secretion levels obtained in the strain expressing allele *cof1-21* (Figure 4.3.2B). These results suggest that removal of mtDNA (generation of so called petite strains), and so a complete loss of respiratory capacity, led to the shutdown of Gluc expression directed by the mating factor signal sequence (Figure 4.3.2A). This significant reduction in rP expression was not observed in petite strains in which cofilin had been mutated, yet when removing mitochondrial DNA in the wild type this caused a highly significant loss in almost all GLuc expression in contrast to what was observed in the cofilin mutant allele strains (Figure 4.3.2D). Mutant allele *cof1-10* was the only mutant stain (already with low mitochondrial function) which had little change to GLuc secretion in the petite strain while mutant

strain *cof1-4, cof1-21* and *cof1-22* had shown a 50% loss or more in GLuc secretion with loss of mtDNA (figure 4.3.2C). *Cof1-10* is part of class II with impaired mitochondrial function, therefore creating a petite had less repercussions for the cell compared to all other strains. This data strongly suggests signalling between cofilin and the mitochondria are effecting rP production due to striking result in wild type, yet no loss to this extent seen in mutant alleles. This evidence suggested that changes to cofilin are buffering the effects on protein production by the loss of mtDNA; this may involve alterations to the signalling of cofilin effecting rP production. Other data also suggests that the MAPK signalling TF, Ste12 may interact with cofilin due to altered Ste12 activity in strains with enhanced mitochondrial function (see section 4.5.3).

We wanted to test rP production with the killer toxin assay in the same mutate allele's as with the GLuc production, as another recombinant protein (Figure 4.4B). In this assay, we found a reduction of toxin in mutant allele's *cof1-10, cof1-18,* and *cof1-22* where mutant allele *cof1-4* and *cof1-21* had shown the same GLuc production as the wild type in comparison (Figure 4.3.2C). Generally, we found little difference between the control and petite strains in the killer toxin assay with the exception of mutant allele *cof1-22* which showed an increase in secretion with mtDNA removed (Figure 4.4). These findings are in contrast to what was found using the GLuc expression construct, and suggest the use of different signal sequences have a profound effect upon the expression and secretion of different rP proteins. The most striking result was that the petite strain expressing *COF1* still produced and secreted the killer toxin, while GLuc expression had been supressed in this strain. However, further experiments to confirm this would require that the signal sequences used for GLuc and Killer toxin be swapped. The expected result would be that Killer toxin production would be supressed in the presence of the mating factor signal sequence when mitochondrial function is lost and that GLuc expression would be similar to that of wild type when directed by the killer toxin pre-sequence.

Ste12 controls the mating response when cells are under stress, such as lack of nutrients, where yeast sporulate in search for nutrients as these specialised cells are highly resistant to environmental stress (Mata *et al.*, 2002; Neiman, 2011). Therefore, we believe that these change to Ste12 signalling, will alter protein expression/secretion as this is driven by the mating factor targeting sequence. This may explain why we see a difference in the killer toxin assay and GLuc expression. It would be interesting to repeat the killer toxin assay but using the alpha factor sequence for secretion. Through western blotting, this model has provided us a means of comparing expression between yeast strains of GLuc trapped in the cell and what was secreted. Nevertheless, due to altered Ste12 levels caused by mutations in cofilin, using the alpha factor signal sequence causes conflict in understanding the changes to GLuc expression. Therefore this signal sequence is not the most appropriate, an alternative

would be to use a signal sequence from another yeast protein which is secreted well and not affected by altered Ste12 signalling, such as invertase, used in breaking down sucrose.

4.5.2   Resistance to rapamycin in cofilin mutant alleles

Due to previous studies linking the control of mitochondrial biogenesis by TORC1 signalling through post-transcriptional control (Bonawitz *et al.*, 2007), we tested rapamycin resistance in strains expressing mutant alleles of cofilin (Hardwick *et al.*, 1999; Laplante and Sabatini, 2009). We identified that cofilin mutant strains with increased mitochondrial biogenesis have an increased resistance to rapamycin (Figure 4.5). This resistance may suggest altered TORC1 signalling, however we found no evidence that cofilin mutant strains led to changes in Sch9 phosphorylation that would suggest this. Micro array data from previous work in the Gourlay lab comparing strains expressing alleles *COF1* to *cof1-6* also revealed an upregulation of an array of ATP-binding cassette (ABC) transporters. This upregulation is believed to be caused by the dramatic increase in respiratory function triggering a retrograde signal (Hallstrom and Moye-Rowley, 2000; Kotiadis *et al.*, 2012). To test if drug pump expression were responsible for resistance to rapamycin, we grew a selection of mutant alleles in rapamycin with FK506 which inhibits the major drug pump *PDR5* (Egner *et al.*, 1998; Hendrych *et al.*, 2009) (Figure 4.6.2). Our findings suggest that unlike the documented resistance to Itraconazole exhibited in cofilin mutant strains with elevated mitochondrial activity (Kotiadis *et al.*, 2012) rapamycin resistance is not conferred by PDR5.

For further confirmation that these drug pumps are not responsible for the rapamycin resistance, was obtained by deleting the transcription factor *PDR1* which regulates the expression of a number of ABC transporter genes. Deletion of PDR1 did not lead to changes on resistance to rapamycin in cofilin mutant strains. However the possibility remained that the expression of other drug pumps may control rapamycin resistance. To identify the drug pumps for which rapamycin may be a substrate we tested an array of strains from our deletion collection with drug pumps or TF that control them removed. These data suggested that the ABC transporters *PDR10* and *PRD18,* and the TF *PDR8* may be involved in the regulation of rapamycin resistance. In support of this *PDR10* levels were increased in cells expressing the cofilin mutant allele *cof1-6,* which displays resistance to rapamycin (Kotiadis *et al.*, 2012). *PDR10* transcription is known to be controlled by both *PDR1* and *PDR3.* However, deletion of PDR1 in resistant cofilin mutant strains did not sensitise cells to rapamycin (Figure 4.9). In addition, the deletion of PDR3 did not lead to rapamycin sensitivity. One possibility is that *PDR8* or another transcription factor controls *PDR10* and *PDR18* levels and that these modulate resistance to rapamycin. This would require further investigation.

4.5.3 Cofilin mutant alleles have altered MAPK signalling effecting rP production

Microarray data from previous work in the Gourlay lab had also shown that MAPK signalling components were upregulated in strains expressing allele *cof1-6*. In particular the MAPK components that were upregulated were components of the Mating and filamentous growth pathways that activate STE12 and STE12/TEC1 signalling respectively (Widmann and Gibson, 1999; Saito, 2010). Changes in MAPK signalling in cells expressing mutant cofilin alleles implicate Cofilin in a role to link environmental signals to physiological responses.

There is considerable reported crosstalk within MAPK signalling pathways. Therefore, we investigated which MAPK pathways were altered when cofilin was mutated. The expression of phosphorylation of the stress activated MAPK, Hog1, were tested (Alepuz *et al.*, 2001; Engelberg *et al.*, 2014). No significant differences were observed in Hog1 phosphorylation in cofilin mutant strains suggesting that this pathway is not promiscuously activated by cofilin mutation. Some phosphorylated Hog1 was detected in *cof1-8* cells during log phase growth. This mutation changes a motif in cofilin that contains a putative MAPK docking site. The level of activation was low but may warrant further investigation. As microarray studies suggested a linked between cofilin and the mating/filamentous growth pathways we examined Ste12 activity using an established reporter system. Data revealed very high levels of Ste12 activity in mutants expressing allele's *cof1-4, cof1-12* and *cof1-19* in class I suggesting increased mitochondrial function may have an effect on Ste12 activity (Figure 4.12). When comparing Ste12 activity in all cofilin mutant strains to rapamycin resistance we observed a positive correlation, suggesting a link between Ste12 levels and rapamycin resistance. To investigate the link between Ste12 and rapamycin resistance further we deleted *STE12* in strains with high MAPK activity and test their rapamycin resistance. Our data had demonstrated that the loss of *STE12* led to a further increase in rapamycin resistance across all strains. This suggests that targets of STE12 may play a role in the regulation of rapamycin resistance, or may regulate the cells TORC1 signalling properties. It would be interesting for example to assess the levels of PDR10 in cells lacking *STE12*.

Due to high Ste12 activity which related to strains with high mitochondrial function, we also tested if removing this gene would affect GLuc expression in mutant alleles with varied Ste12 activity. Although there was no change to the native form of GLuc with *STE12* deleted (Figure 4.15.2), an increase was observed in the incorrectly processed forms of GLuc that appear on western blots in the pellet suggesting that Ste12 signalling has a role in the processing of rP (Figure 4.16). It may be the case that by deleting *STE12*, there a loss of control in the MAPK mating pathway which translates to an increase in the signal to produce alpha factor and so in this case expression of GLuc. However as we observed

only a modest increase in secretion it seems likely that the general secretory pathway was unable to cope. It would be necessary to increase the capacity of the secretory system to allow the increase in protein produced to be correctly folded and secreted. Future work would involve increasing the capacity of the secretory pathway through the overexpression of chaperones to increase proper protein folding. For example, the overexpression PDI, an enzyme needed for disulphide formation which has been proven to benefit rP production (Inan, Aryasomayajula, Sinha, & Meagher, 2006; Wittrup, 1995). In the yeast, *P.pastoris*, overexpression of PDI enhanced the secretion of the protein *Necator americanus* (Na-ASP1), a potential vaccine against hookworm infections (Inan *et al.*, 2006).

We observed a loss of killer toxin secretion in mutant allele *cof1-4Δste12* compared to the control strain *cof1-4* (Figure 4.17B)*. The* cofilin mutant allele *cof1-22* had an increase in killer toxin secreted with *Δste12* compared to the control strain. These results vary compared to GLuc production, again confirming results of GLuc production are specific to that construct containing the mating pheromone signal sequence.

4.5.4 Conclusion

This chapter has highlighted the connections between the control of MAPK signalling and mitochondrial function by the actin binding protein Cofilin and rP secretion. This work strongly implicates the regulation of STE12 in the control of expression of rP protein that uses the mating factor signal sequence. Cofilin and mitochondrial function appear to be important for the regulation of MAPK signalling through STE12 and so can influence the expression of rP molecules that use the mating factor signal sequence. This work should be furthered by enhancing the capacity of the secretory pathway in cells lacking STE12, which led to a significant increase in expression of the rP Gluc. This may be achieved through the overexpression of the ER protein, PDI, improving the secretion of GLuc. It may be possible to significantly enhance rP signalling using this approach.

STE12 expression also appears to be linked to resistance to the drug rapamycin. We suggest that the rapamcyin resistance was not due to hyper active TOR signalling even though previous micro array data from the Gourlay lab has pointed towards this pathway but due to the upregulated drug pumps PDR10, PDR18 and TF PRD8.

Chapter 5

Investigation and Manipulation of TOR Signalling for Altered Recombinant Protein Production in Yeast

5.1 Introduction to the role of TOR signalling in recombinant protein production

The improvement of recombinant protein production is a difficult task given the complexity of protein synthesis, protein processing and folding and secretion (Hou *et al.*, 2012). Engineering a cell to over express a protein may not be straight forward, for example elevation of a particular rP may be toxic (Graf *et al.*, 2009). Environmental conditions or cell responses to stress, protein folding/aggregation and secretion can also contribute to the efficiency of rP production (Gasser *et al.*, 2007). However, despite the complexities of cell biology, it may be possible to improve rP production by manipulation of central pathways that co-ordinate environmental signalling with growth, rather than just improving one particular cell trait (Dreesen and Fussenegger, 2011).

The TORC1 (target of rapamycin) signalling network plays an important role in integrating environmental signals with growth. A brief description of this pathway and its control is provided in the introduction chapter of this thesis (see section 1.3.5). In view of TOR being a described as a master regulator of signalling, protein synthesis, ribosome biogenesis and proliferation within the cell, here it was investigated whether the manipulation of TORC1 signalling could be employed to enhance recombinant protein (rP) production in a yeast expression system. To achieve this, a screening approach was employed whereby *Gaussia* luciferase expression and secretion was assayed in a library of yeast strains deleted for individual genes within the TORC1 network. The genes that were specifically investigated are described in figure 5.1 and table 5.1.
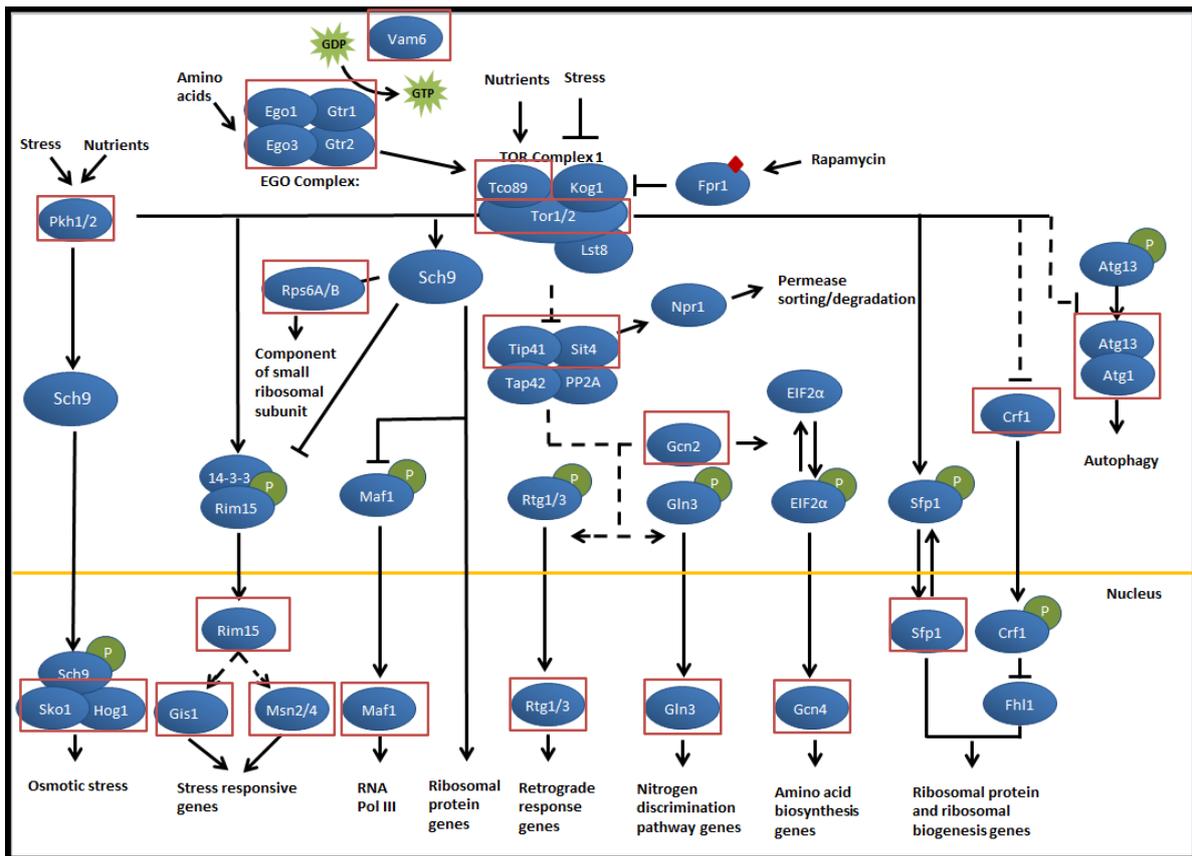
Figure 5.1: The TORC1 network in yeast. Upstream and downstream regulators of TORC1, most of which are used in the initial screen discussed in this chapter (identifiable by red box). The green P indicates phosphorylation. Key above shows arrows representing interactions. Adapted from Smets et al., 2010. The red boxes highlight genes studied in this chapter.

Table 5.1: List of genes investigated and roles in TORC1, and grouped according to their roles.

Gene	Function
SIT4	functions in the G1/S transition of the mitotic cycle
TIP41	response to DNA replication stress
PKH1	required for maintenance of cell wall integrity
PKH2	required for maintenance of cell wall integrity
SKO1	cytosolic and nuclear protein involved in osmotic and oxidative stress responses
HOG1	MAPK involved in osmoregulation
RTG1	Retrograde signalling
RTG3	Retrograde signalling
MSN2	TF that regulates the general stress response
MSN4	TF that regulates the general stress response
RIM15	Stress response protein kinase
GIS1	Stress response TF
GTR1	Component of EGO complex in amino acid sensing, signals directly to TORC1
GTR 2	Component of EGO complex in amino acid sensing, forms heterodimer with Gtr1
EGO1	Component of EGO complex in amino acid sensing, acts as an anchor to the vacuole
EGO3	Component of EGO complex in amino acid sensing, binds with Ego1
VAM6	Vacuolar protein and GEF for GTR proteins, role in vacuolar membrane fusion
ATG1	Autophagy gene, vesicle formation in autophagy, binds to Atg13
ATG13	Autophagy gene, regulatory subunit of Atg1 signalling complex
TOR1	subunit of TORC1
TCO89	component of the TOR complex 1, role in cell wall integrity
FRP1	Encodes FKBP12, rapamycin target in TORC1
RPS6A	Protein component of the small (40S) ribosomal subunit
RPS6b	Protein component of the small (40S) ribosomal subunit

CRF1	role in repression of RP genes
SFP1	Regulates transcription of ribosomal protein and biogenesis genes
GCN2	Protein kinase, phosphorylates alpha-subunit of eIF2 in response to starvation
GCN4	transcriptional activator of amino acid biosynthetic genes
GLN3	TF involved in positively regulating genes subject to nitrogen catabolite repression (NCR)
NPR1	Permease sorting and degradation, possibly involved in ribosome biogenesis
MAF1	negative regulator of RNA polymerase III

TORC1 signalling provides the cell with a means of monitoring, responding to and controlling stress response genes that contribute to processes such as osmotic stress response, the retrograde response, loss of nutrition and energy depletion and DNA damage which leads to apoptosis (Proud, 2007). Evidence supports the idea that manipulating expression levels of upstream and downstream TORC1 targets may affect rP production. For example CHO (Chinese hamster ovary) cells engineered to constitutively overexpress mTOR in one particular study found increased cell size, protein content, viability and proliferation. These cells exhibited reduced mortality due to the action of TOR in increasing cell robustness to sub-optimal culture conditions seen with oxygen and growth factor limitations (Dreesen and Fussenegger, 2011). In cells with the co-overexpression of Bcl-2 (inhibiting apoptosis) and pro-autophagy by Becinl-1 (Atg6/Vps30 yeast orthologue) a longer culture period allowing autophagy to remain within the homeostatic range was observed, reflecting the importance of regulating autophagy during rP production (Pattingre *et al.*, 2008; Lee *et al.*, 2013). This is further evidence of how controlling TORC1 improves key bioprocesses - relevant characteristics discussed above resulting in engineered CHO cells with improved secretion of therapeutic IgGs with a four-fold increase over the wild type expression levels (Dreesen and Fussenegger, 2011).

External stresses such as temperature, nutrient limitation and pH, along with intrinsic stress from protein expression can limit rP production (Ferrer-Miralles *et al.*, 2009). When *P.pastoris* was subjected to sub-optimal temperature (20℃), this resulted in a down regulation of stress response genes. This also led to an increase in secretion of a Fab antibody fragment due to enhanced transcription of components of secretory transport and the ER associated degradation (Gasser *et al.*, 2007). In *S.cerevisae*, growth in low temperatures results in an TORC1 mediated upregulation of ribosome biogenesis genes increasing protein synthesis (Tai *et al.*, 2008). Further evidence for the involvement of TORC1 in rP production was reported by Kitagawa et al. (2011) who identified that the transcription factor SFP1, which controls the TORC1 and PKA dependent expression of ribosomal biogenesis genes via the localisation of Sfp1, is involved in the enhancement of cellulose protein production (Marion *et al.*, 2004; Kitagawa *et al.*, 2011).

Through TORC1 regulation, translational activity is increased when CHO cultures are continually fed nutrients leading to further growth and productivity (Huang *et al.*, 2010; Edros *et al.*, 2014). A recent paper which studied polysome profiling with rapamycin treatment in CHO culture found a shift in the proportion of polysomes (or polyribosomes) towards monosomes with a 30% decrease in maximal growth rate compared to the batch culture with a supply of nutrients activating the TOR pathway. Targeted quantification of the mRNA of heavy and light chain antibodies showed up to 5-fold higher specific translation due to the increase (up to 2-fold) in global translation and increased cellular growth

with nutrients supplied (Courtes *et al.*, 2013). The examples given above are evidence supporting the hypothesis that TORC1 intervention may present a viable approach to manipulate rP production, and to identify new potential avenues outside stress and death control for manipulating the cell for improved recombinant protein synthesis.

Results

5.2 A TOR screen to investigate alterations in recombinant protein production

5.2.1 Effects of rapamycin on growth and GLuc expression

Rapamycin binds to FKBP which in turn inhibits TORC1 signalling causing decreased protein synthesis and ribosomal biogenesis with a reduction in growth (Dubouloz *et al.*, 2005). We therefore tested how rapamycin treatment would affect rP expression in wild type yeast cells (BY4741). To achieve this we introduced a plasmid expressing the *Gaussia* luciferase gene (GLuc) from the organism *Gaussia principes* (described in chapter 3) and measured expression and secretion during log phase growth by western blotting (Tannous *et al.*, 2005).

To find the optimum rapamycin concentration, a range of concentrations were investigated and the effect on growth monitored by absorbance using an automated plate reader (see Figure 5.2a). This was undertaken to identify the lowest concentration at which rapamycin would measurably slow growth and rP production while maintaining cell viability so GLuc production could still be detected by western blot. From these experiments it was determined that a concentration between 2 and 5 ng/ml was suitable for further use (Figure 5.2.1-5.2.2).

A)

B)

C)

D)
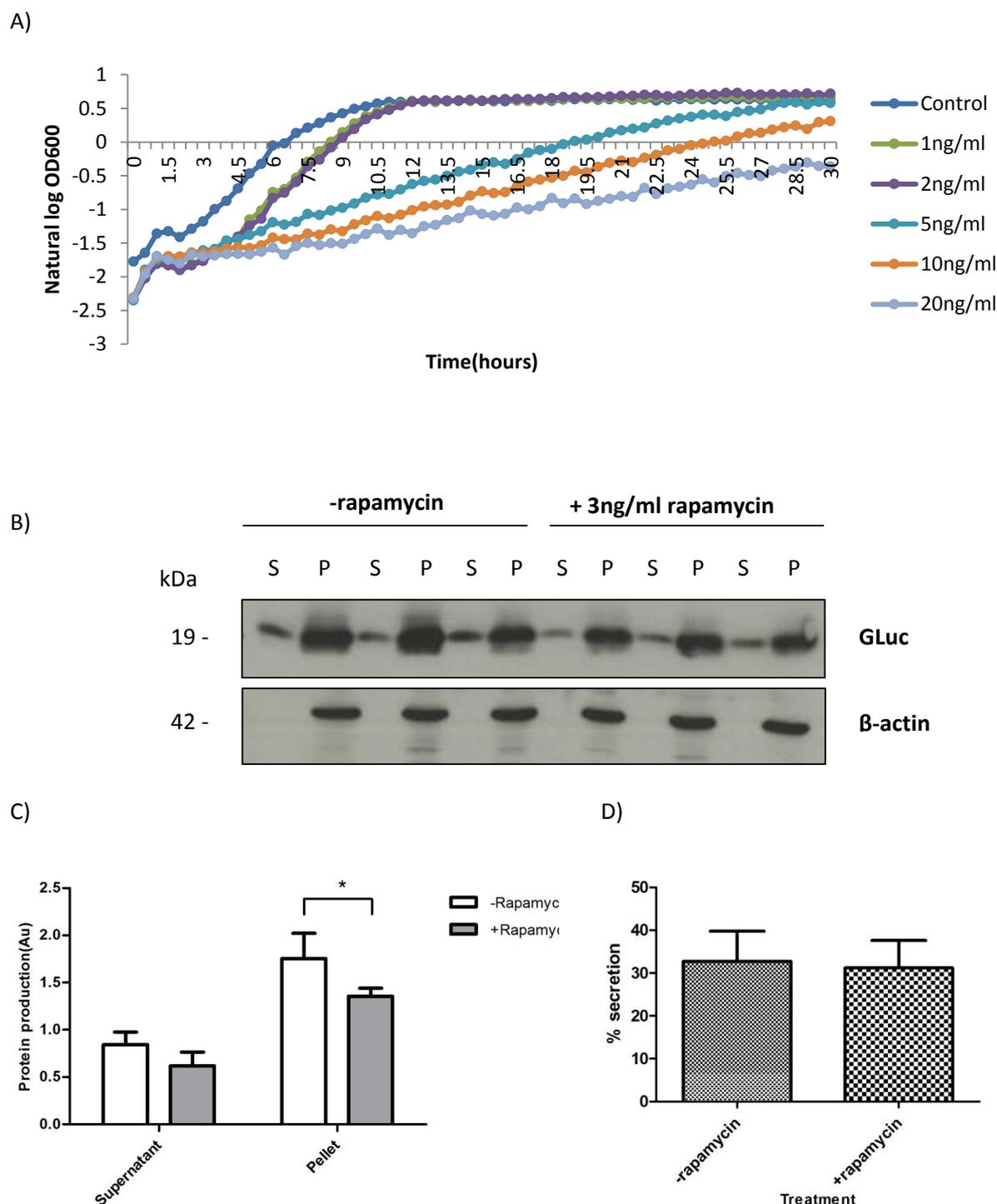
Figure 5.2.1 Effect of rapamycin treatment on GLuc expression in wild type cells. A) Investigating effects of varied rapamycin treatment on growth of wild type (BY4741) (n=1). B) Blot of GLuc and β-actin expression as control with and without 3 ng/ml rapamycin (n=3). C) Quantification of GLuc and actin expression as a loading control in the supernatant and pellet using the free software Image J. D) Percent GLuc secreted with and without 3 ng/ml rapamycin treatment. Cells were grown overnight in YPD containing either DMSO or rapamycin, and reinoculated in media at the cell count 1×10^6 cells/ml with DMSO or rapamycin and grown to the cell count 1×10^7 cells/ml. Protein was extracted from the pellet using the yeast whole cell quantitative method. Errors bars represent standard deviation. Statistical analysis was performed using two way ANOVA (*$P<0.05$).

A)
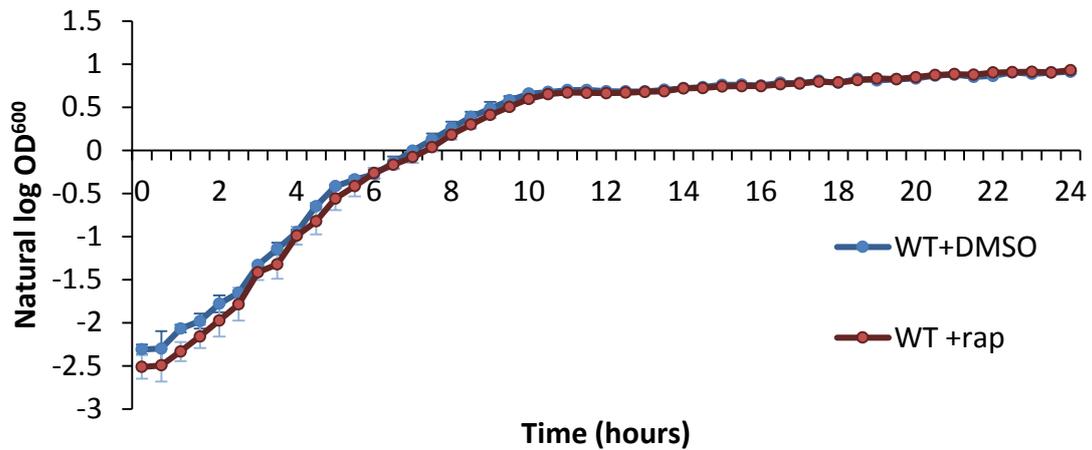
B)
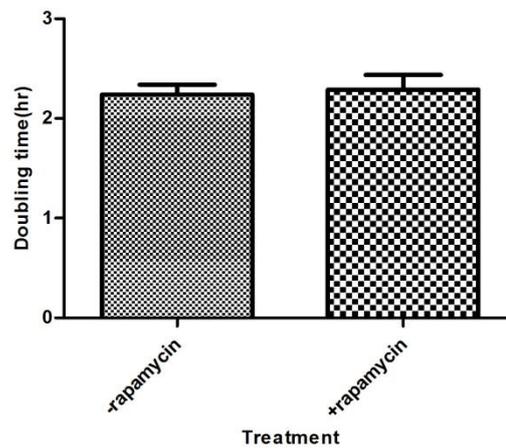
Figure 5.2.2 Effect of rapamycin treatment on growth in wild type cells with 3 ng/ml rapamycin. A) Growth of the wild type with and without 3 ng/ml rapamycin treatment. B) Calculated doubling time of wild type cells with and without rapamycin treatment. Cells were grown overnight in YPD containing either DMSO or rapamycin, and reinoculated in media at the cell count 1×10^6 cells/ml with DMSO or rapamycin and grown to the cell count 1×10^7 cells/ml. Protein was extracted from the pellet using the yeast whole cell quantitative method. Errors bars represent standard deviation. Statistical analysis was performed using two way ANOVA (*P<0.05).

Following the addition of more than 5 ng/ml rapamycin there was a strong effect on growth with continued growth inhibition as the concentration is doubled (Figure 5.2.1A). From this growth analysis we decided to use a concentration of 3 ng/ml so that there was still an effect on growth but the cells were still viable and produced rP. This concentration of rapamycin was used to test the effects of GLuc expression during log growth where we observed a decrease in GLuc protein production observed on the western blot (Figure 5.2.1C). However, after quantification we observed no significant change to secretion with 3 ng/ml rapamycin treatment (Figure 5.2.1D) but a decrease in GLuc trapped in the pellet (Figure 5.2.1C). When calculating the doubling time of the wild type with and without 3 ng/ml rapamycin treatment (Figure 5.2.2A), there was no significant change to growth (Figure 5.2.2B) suggesting rP production can be reduced by the inhibition of TORC1 signalling at this concentration without affecting cell growth.

5.2.2 A TOR screen to identify genes that alter recombinant protein production in yeast

To identify specific targets, both upstream and downstream of TORC1 which can modulate rP production, we screened all available yeast strains deleted for each non-essential gene that has been identified within the TOR signalling network. GLuc expression and secretion was tested during log phase of growth by western blot. It should be noted that while strains did achieve log phase growth within four hours of sub-culture (determined by cell counting) there were some obvious differences in growth rate associated with the deletion. This represents a limitation of the strategy as in these cases samples were in early log or late log phase. Due to the large numbers of samples, it was not feasible to wait for strains to reach the same cell count. However, all results obtained were reproducible and this initial screen was used to identify putative candidate genes whose deletion resulted in altered rP production (Figure 5.3.1-5.3.6).
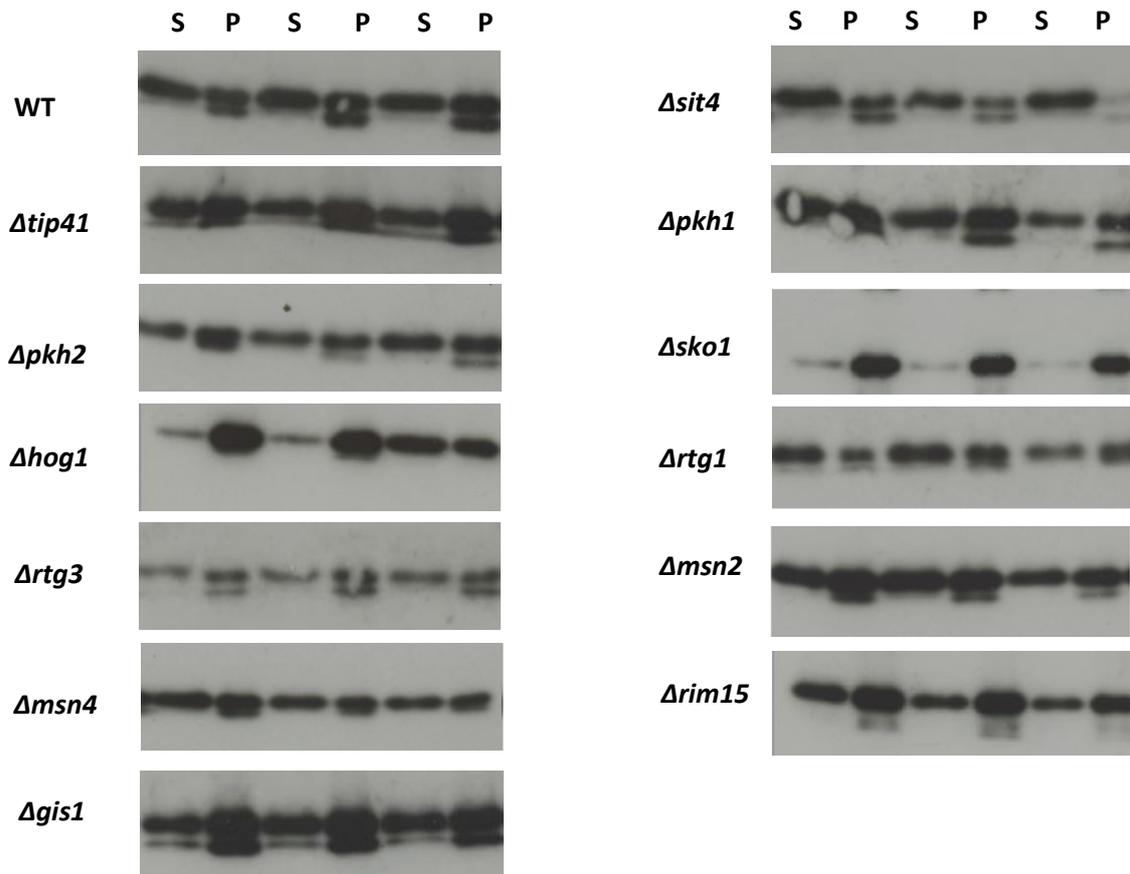
Figure 5.3.1 Results of GLuc expression in yeast cells deleted for components of the TOR signalling network. Cells were grown in selective media overnight and re-inoculated to an OD_{600} of 0.3 in YPD and left to grow into early log (roughly 4 hrs). A whole cell protein extraction method was used to test GLuc found in the supernatant and pellet (n=3). Blots showing GLuc expression in the supernatant (S) and pellet (P). Errors bars represent standard deviation. Statistical analysis was performed using one way ANOVA test with significance annotated comparing what was detected in the supernatant of each strain to the wild type, the same for pellet against the wild type (**P<0.05, **P<0.01 ***P<0.001).

Figure 5.3.2 Results of GLuc expression in yeast cells deleted for components of the TOR signalling network. Cells were grown in selective media overnight and re-inoculated to an OD_{600} of 0.3 in YPD and left to grow into early log (roughly 4 hrs). A whole cell protein extraction method was used to test GLuc found in the supernatant and pellet (n=3). Blots showing GLuc expression in the supernatant (S) and pellet (P). Errors bars represent standard deviation. Statistical analysis was performed using one way ANOVA test with significance annotated comparing what was detected in the supernatant of each strain to the wild type, the same for pellet against the wild type (**P<0.05, **P<0.01 ***P<0.001).

A)

B)

C)

D)

Figure 5.3.3 Quantified results of GLuc expression in yeast cells deleted for components of the TOR signalling network. Cells were grown in selective media overnight and re-inoculated to an OD_{600} of 0.3 in YPD and left to grow into early log (roughly 4 hrs). A whole cell protein extraction method was used to test GLuc found in the supernatant and pellet (n=3). A) Quantification of GLuc expression in the wild type using the software Image J. B-D) GLuc expression in TOR screen as a factor of the wild type. Errors bars represent standard deviation. Statistical analysis was performed using one way ANOVA test with significance annotated comparing what was detected in the supernatant of each strain to the wild type, the same for pellet against the wild type (**$P < 0.05$, **$P < 0.01$ ***$P < 0.001$).

A)

B)

C)

D)
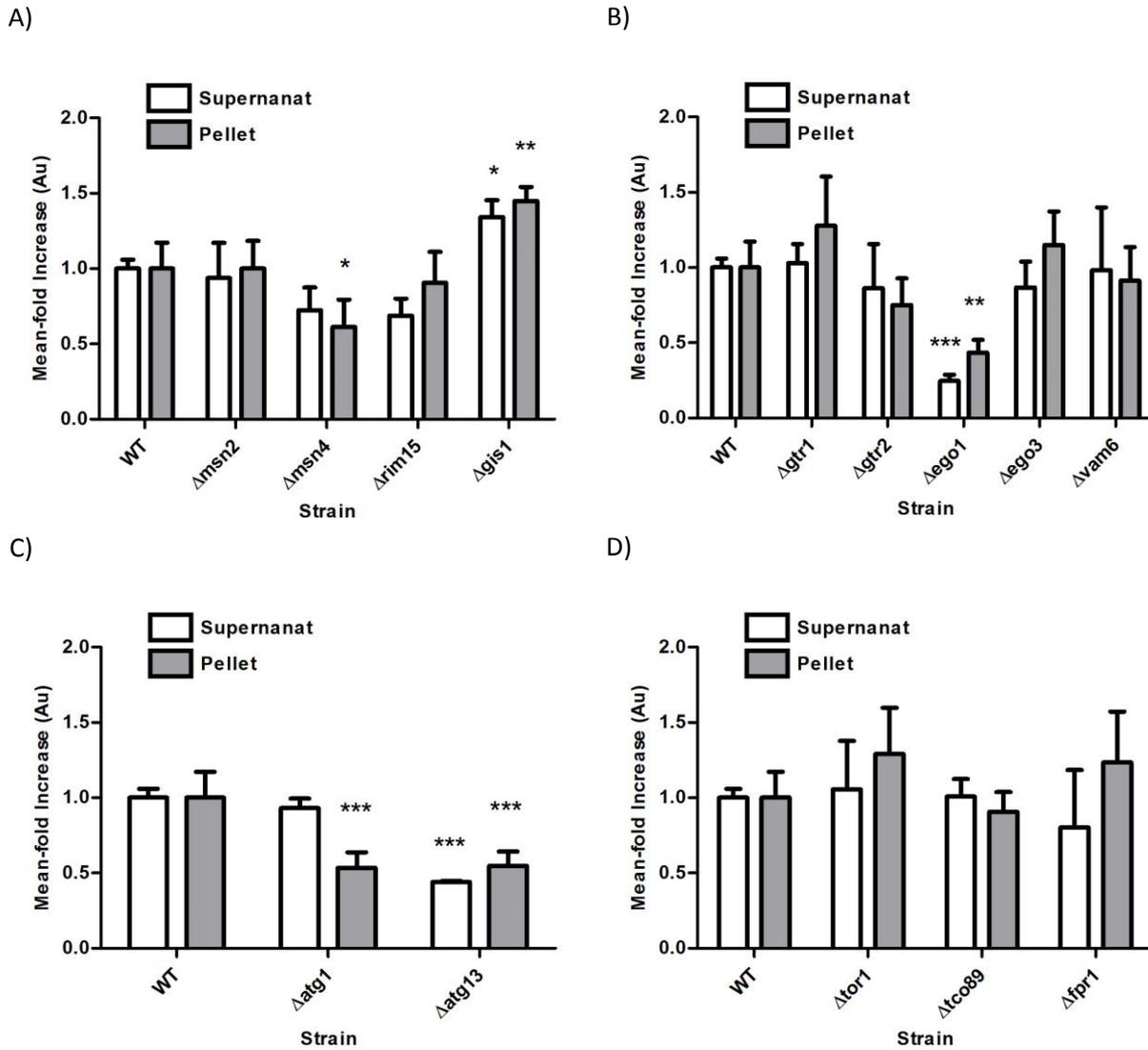
Figure 5.3.4 Quantified results of GLuc expression in yeast cells deleted for components of the TOR signalling network. Cells were grown in selective media overnight and re-inoculated to an OD_{600} of 0.3 in YPD and left to grow into early log (roughly 4 hrs). A whole cell protein extraction method was used to test GLuc found in the supernatant and pellet (n=3). A-D) GLuc expression in TOR screen as a factor of the wild type. Errors bars represent standard deviation. Statistical analysis was performed using one way ANOVA test with significance annotated comparing what was detected in the supernatant of each strain to the wild type, the same for pellet against the wild type (**$P<0.05$, **$P<0.01$ ***$P<0.001$).

A)

B)

C)

D)

Figure 5.3.5 Quantified results of GLuc expression in yeast cells deleted for components of the TOR signalling network. Cells were grown in selective media overnight and re-inoculated to an OD_{600} of 0.3 in YPD and left to grow into early log (roughly 4 hrs). A whole cell protein extraction method was used to test GLuc found in the supernatant and pellet (n=3). A-D) GLuc expression in TOR screen as a factor of the wild type. Errors bars represent standard deviation. Statistical analysis was performed using one way ANOVA test with significance annotated comparing what was detected in the supernatant of each strain to the wild type, the same for pellet against the wild type (**P<0.05, **P<0.01 ***P<0.001).

A)

B)

Figure 5.3.6 Quantified results of GLuc expression in yeast cells deleted for components of the TOR signalling network. Cells were grown in selective media overnight and re-inoculated to an OD_{600} of 0.3 in YPD and left to grow into early log (roughly 4 hrs). A whole cell protein extraction method was used to test GLuc found in the supernatant and pellet (n=3). A) GLuc expression in Δmaf1 screen as a factor of the wild type. B) Percentage GLuc secreted in screen from western blots shown in 5.3.1A. Errors bars represent standard deviation. Statistical analysis was performed using one way ANOVA test with significance annotated comparing what was detected in the supernatant of each strain to the wild type, the same for pellet against the wild type (**$P<0.05$, **$P<0.01$ ***$P<0.001$).

When testing this background strain of yeast, in the wild type we found that approximately 45% of GLuc product was secreted (see Figure 5.3.6B). From the pellet, a doublet was observed; at 19 kDa was the GLuc mature product with the lower band showing some possible degradation product. An interesting observation was that not all strains produced this double band as the wild type did (Figure 5.3.1-5.3.2).

The deletion of a number of genes in the TOR signalling network lead to variation in GLuc expression with most showing a decrease in the protein detected. A summary of these results is provided below in Table 5.2 listing which were significantly higher or lower producers of GLuc when the specified gene was removed (Figure 5.3.1-5.3.6).

Table 5.2 High and low producers of GLuc detected in the supernatant and pellet with deleted components of the TORC1 pathway. This is a summary of data from figure 5.3.3B-5.3.6A above placing strains within their grouped roles. Stars represent their statistical significance (**P<0.05, **P<0.01 ***P<0.001) Gene names in bold had shown reduced secretion compared to the wild type as a percentage of total GLuc calculated. S (supernatant), P (pellet).

Increase in rP production:

Gene	S	P	Role in TORC1 signalling
TIP41		*	response to DNA replication stress/subunit of complex signalling
GIS1	*	**	Stress response TF

Decrease in rP production:

Gene	S	P	Role in TORC1 signalling
SIT4		**	response to DNA replication stress/subunit of complex signalling
PKH2	*		cell wall integrity and osmoregulation
SKO1	***		
HOG1	**		
RTG1	*	***	Retrograde signalling
RTG3	***	***	
MSN4		*	general stress response
EGO1	***	**	amino acid sensing
ATG1		***	Autophagy
ATG13	***	***	
RPS6A	***	***	ribosomal subunit
RPS6B	*	*	
CRF1	***	***	Ribosomal protein and biogenesis
SFP1	**		
GCN4	*		Nitrogen discrimination
GLN3	***	*	
NPR1	**		Permease sorting/degradation

With alterations in the expression to specific genes within the TORC1 pathway, varied alterations in GLuc production were observed, some resulting in a total loss in GLuc expression detected in the supernatant and pellet whilst in other cases there was an increase. In other cases a change to GLuc was detected in either the pellet or supernatant, but not both relative to the wild type. This data is summarised from figures 5.3.1/2/3/4 in table 5.2. These strains were placed into groups depending on their described role in the cell.

The deleted genes causing an increase in observed GLuc production included *TIP41*, coding for the protein in the complex downstream of TORC1 which had an increase in GLuc trapped in the pellet in comparison to the wild type. The stress response TF *GIS1* when deleted caused an increase in total GLuc production detected in both the supernatant and pellet compared to the wild type. Surprisingly, compared to the wild type, the other component of the complex below TORC1 when the gene coding for *SIT4* was deleted, a decrease in GLuc was detected in the pellet in contrast to the deletion of *TIP41.* Multiple components of the TORC1 complex were deleted and GLuc production determined, however, there was no significant change to quantified GLuc expression in either the supernatant or pellet compared to the wild type (Figure 5.3.1A).

A reduction in GLuc detected was observed in the supernatant compared to the wild type in removal of the cell wall integrity and osmoregulation genes *PKH2* (however no change with removal of *PKH1*), *HOG1* and *SKO1* which incidentally gave a significant loss in the percentage of GLuc secreted of the total GLuc quantified (all samples had the same cell number). Other stress pathways effecting GLuc production with genes removed include the retrograde genes *RTG1/3* causing a total loss in GLuc production as there was less GLuc detected in both the supernatant and pellet in comparison to the wild type. However, the stress responsive gene MSN4 when deleted caused a loss to GLuc expression detected in the pellet yet no change in *Δmsn2* or *Δrim15* deletions.

Interestingly, the data suggest a role for the EGO complex (with a role in amino acid sensing) in rP production as deletion of *EGO1*, which is responsible for anchoring the EGO complex to the vacuole, caused a loss in total GLuc production as there was significantly less GLuc detected in both the supernatant and pellet compared to the wild type. However, when comparing the other strains Δgtr1, Δgtr2, Δego3 and Δvam6, also involved in this complex to the wild type, no significant changes to GLuc expression levels in the pellet or supernatant were observed. Yet when comparing GLuc expression in Δgtr1 to its heterodimer removed Δgtr2, a significant change in GLuc expression detected in the pellet was observed. Moreover, when comparing GLuc expression in Δego1 to Δego3, we discovered a significant increase in GLuc expression in both the supernatant and the pellet; all this data signifies the involvement and importance of the EGO complex.

We observed changes to GLuc expression in comparison to the wild type in autophagy deletion strains Δatg1 and Δatg13, where both deletions lead to a reduction in GLuc detected in the pellet yet a reduction in the supernatant in Δatg13 in comparison to the wild type. When comparing the percentage of the total GLuc detected that was secreted, we found that Δatg1 had a 14% increase (Figure 5.3.4B).

TORC1 signalling controls components of the small ribosomal subunit (RPS6A/PRS6B) and regulation of ribosomal proteins and biogenesis (via CRF1 and SFP1) downstream. When these genes were deleted, a significant loss of GLuc detected in the supernatant and pellet was observed (bar SFP1 which just had a decrease in GLuc in the supernatant) in comparison to the wild type. Removal of the nitrogen discrimination gene (*GLN3 and GCN4*) and the removal of control of permeases Gap1 and Tat2 by deletion of *NPR1* all had a negative effect on rP as there was a loss pf GLuc detected in the supernatant in comparison to the wild type (Smets *et al.*, 2010). However, in comparison to the wild type, Δ*GLN3* also exhibited a loss of GLuc expression detected in the pellet.

In conclusion, a range of genes within the TORC1 signalling pathway appear to impact upon the production of GLuc. The roles of the genes that have been identified are varied, however in some cases more than one gene within the same process was identified, for example within the EGO complex or the process of autophagy.

5.2.3 Overexpression of selected TOR targets

From the initial screen we selected a small number of deletion strains for further examination. This was done to confirm reproducibility of the results obtained from the screening process and to determine whether observed changes in rP production or secretion were a result of the loss of the gene in question as opposed to strain background effects.

Therefore we investigated further whether loss of the genes encoding *GTR1* and *GTR2*, which are part of the EGO complex, and their GEF, *VAM6*, led to changes to rP production. The screen (Figure 5.3) had shown differences in expression profile between the removal of the heterodimers Gtr1 and Gtr2. This is an interesting result as they are known to work together in signalling to TORC1 (Binda *et al.*, 2009). Although we detected changes to rP production in EGO1 and EGO3 deletions (these proteins are so far only known to function as an anchor to the vacuole), we were interested in investigating the GTR component of the complex as this directly effects amino acid sensing (Zhang *et al.*, 2012). Despite these changes to the EGO complex, we found no alteration to rP production when

deleting *VAM6* where this is known to act as a GEF to Gtr1 and Gtr2. However, further studies were undertaken on this gene due to its role influencing the EGO complex and TORC1 downstream.

In addition we further analysed *TCO89* encoding the TORC1 component Tco89. Deletion of this gene reliably altered rP production where we found no change to GLuc expression compared to the wild type, but less degradation product suggesting Tco89 may affect stability of GLuc. Little is known about the role of this gene however recent data suggests that Tco89 directly interacts with Gtr1 in signalling to the TORC1 complex and so continued to investigate this gene (Sekiguchi *et al.*, 2014).

Deletion in autophagy genes, *ATG1* and *ATG13* resulted in the loss of rP production, with an increase in the ratio of GLuc found in supernatant to pellet in *Δatg1* of 14% (Figure 5.3). Both proteins work together along with Atg17 (FIP200 in mammalian cells) in initiating autophagy, producing an interesting result when this was measured in log phase where there are still an abundance of nutrients. It has been reported that increased Atg1 alone leads to down regulation of TOR activity which illustrates the importance of the balance of autophagy (Scott *et al.*, 2007). We chose to focus on Atg1 as this is the main coordinator of autophagosome formation with direct signalling to S6K (Sch9 in yeast) in drosophila (Lee *et al.*, 2007; Mizushima, 2010).

To further investigate whether these deleted genes were responsible for the observed changes in rP production, an expression vector was created to re-express each protein in the deletion strains.

5.2.3.1 Measuring mRNA levels with overexpression

In order to check that the vectors generated for re-expression were functional we measured the mRNA levels during log phase growth. mRNA was extracted from deletion strains containing the plasmid overexpressing each gene and mRNA levels measured using qRT-PCR. Measured mRNA levels are presented in figure 5.4 showing mRNA measured relative to the wild type with selected TOR strains overexpressed in their deletion strains.

A)

B)

C)

D)

E)
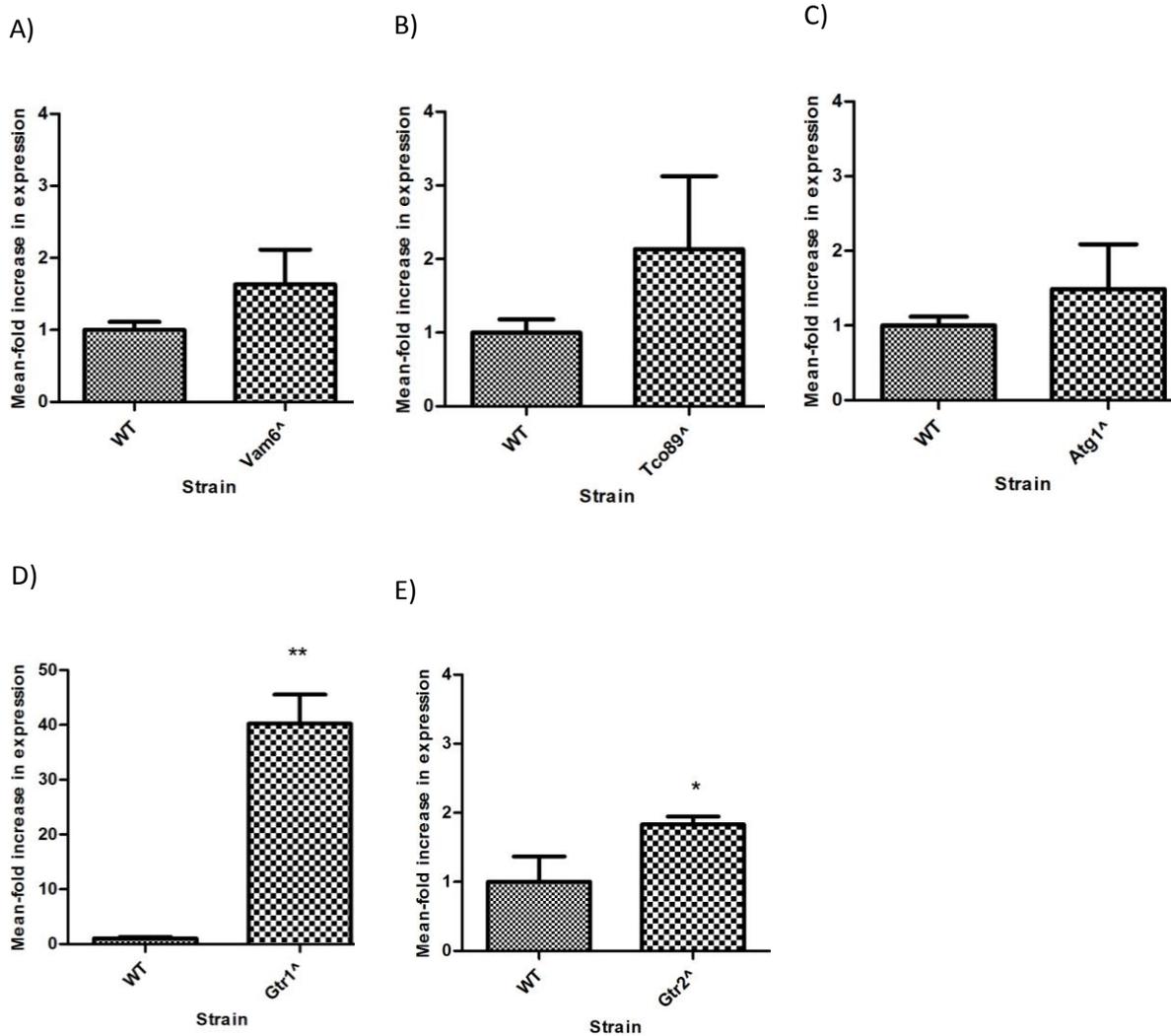
Figure 5.4 Relative mRNA levels of overexpressed *VAM6, TCO89, GTR1, GTR2,* and *ATG1* relative to wild type. A-E) Mean-fold increase in mRNA expression of each gene. mRNA was extracted from strains grown overnight in selection media and re-inoculated in YPD to the cell count 1×10^6 and grown to the cell count 1×10^7 cells/ml. (n=3) Actin mRNA levels were measured and used to normalise the data. Errors bars represent standard deviation.

The data obtained in figure 5.4 shows that mRNA of *GTR1 and GTR2* produced from a plasmid source led to a significant increase in the mRNA level of each gene tested relative to the wild type. *GTR1* showed an exceptionally large increase, yet there was no significant change in mRNA with *TCO89, ATG1* and *VAM6* suggesting their overexpression plasmids did not result in an increase in mRNA amounts.

5.2.3.2 Overexpression of selected genes Δatg1, Δtco89, Δgtr1, Δgtr2 and Δvam6

We wanted to further analyse the changes in rP production observed in *Δatg1, Δtco89, Δgtr1, Δgtr2* and *Δvam6* strains. We also introduced expression vectors to re-express each protein into the appropriate gene knockout strains. These cells were grown into log phase and sampled for GLuc expression in the supernatant and pellet by western blotting. Figure 5.5 shows the results of this test.

A)

B)

C)
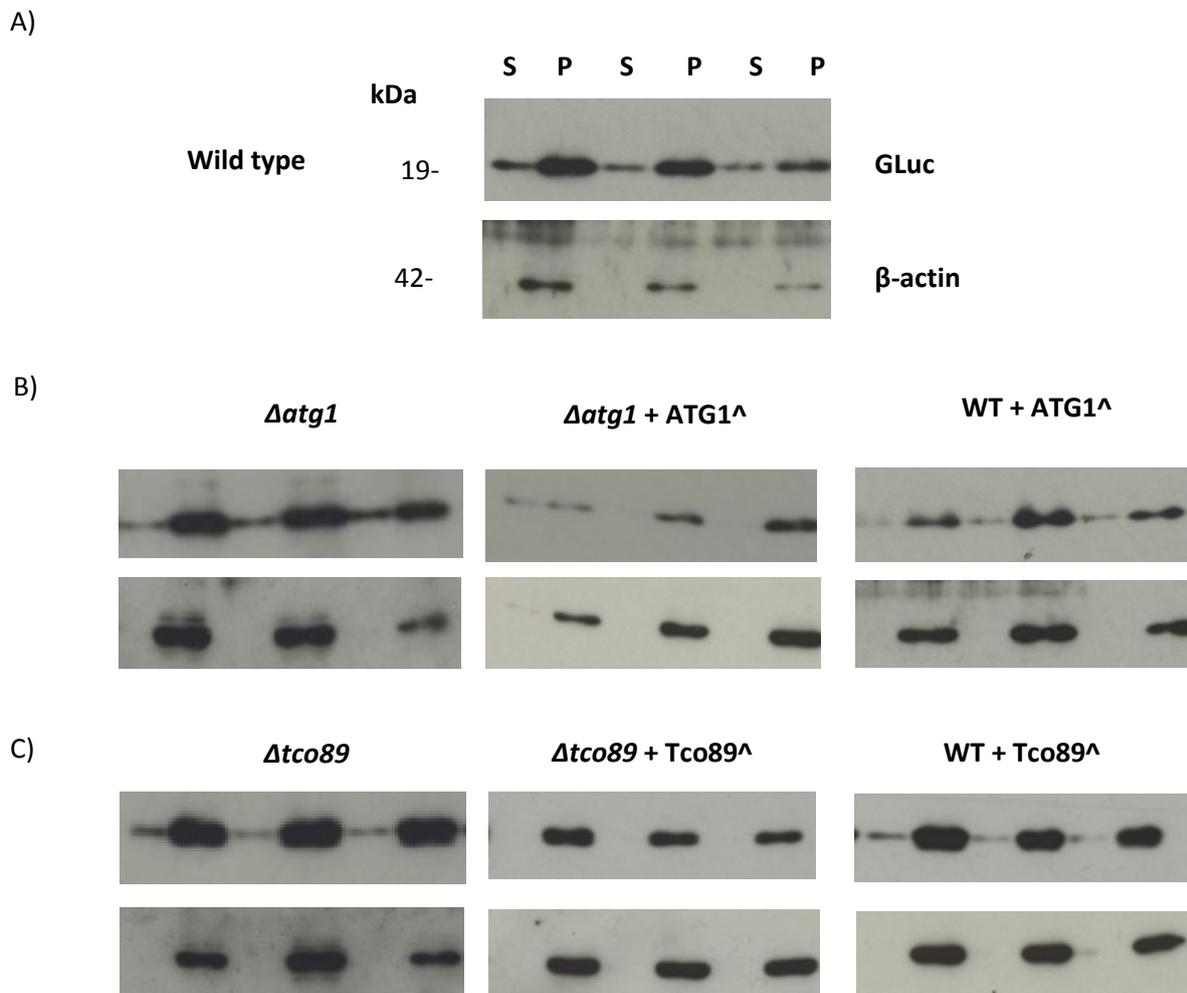
Figure 5.5.1 GLuc expression was assessed in *Δatg1* and Δtco89, transformed with either an empty plasmid (marked +e) or with a vector re-expressing the deleted gene. a) GLuc and actin expression in the wild type as control. b) Blots of GLuc and actin expression of *Δatg1* in the supernatant(s) and pellet (p). C) Blots of GLuc and actin expression of *Δtco89* in the supernatant(s) and pellet (p). Cells were grown overnight in selective media and re-inoculated in YPD at the cell count $1x10^6$ and grown to the cell count $1x10^7$. Protein was extracted from the pellet using the yeast whole cell quantitative method (von der Haar, 2007) (n=3). Expression of GLuc and actin in the western blots were quantified using the software Image J. Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test with significance annotated comparing what was detected in the supernatant of each strain to the wild type, and what was detected in the pellet of each strain to the wild type (*$P<0.05$, **$P<0.01$ ***$P<0.001$).

A)

B)

C)

D)

Figure 5.5.2 GLuc expression was assessed in *Δgtr1, Δgtr2 and Δvam6* transformed with either an empty plasmid (marked +e) or with a vector re-expressing the deleted gene. a) GLuc and actin expression in the wild type as control. B-D) Blots of GLuc and actin expression of *Δgtr1, Δgtr2 and Δvam6* in the supernatant (s) and pellet (p). Cells were grown overnight in selective media and re-inoculated in YPD at the cell count 1×10^6 and grown to the cell count 1×10^7. Protein was extracted from the pellet using the yeast whole cell quantitative method (von der Haar, 2007) (n=3). Expression of GLuc and actin in the western blots were quantified using the software Image J. Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test with significance annotated comparing what was detected in the supernatant of each strain to the wild type, and what was detected in the pellet of each strain to the wild type (*$P<0.05$, **$P<0.01$ ***$P<0.001$).

A)

B)

C)

Figure 5.5.3 GLuc expression was assessed in *Δgtr1, Δgtr2, Δvam6, Δatg1* and *Δtco89* strains transformed with either an empty plasmid (marked +e) or with a vector re-expressing the deleted gene. A) Quantified GLuc expression of *Δgtr1, Δgtr2, Δvam6, Δatg1* and *Δtco89* from Figure 5.5.1-3. B) Quantified Gluc expression relative to the wild type of *Δgtr1* transformed with an empty plasmid, and re-expression in the deletion stain and wild type (Western blot seen in Figure 5.5.1-3). This is also shown in C) with *Δgtr2*. Cells were grown overnight in selective media and re-inoculated in YPD at the cell count 1×10^6 and grown to the cell count 1×10^7. Protein was extracted from the pellet using the yeast whole cell quantitative method (von der Haar, 2007) (n=3). Expression of GLuc and actin in the western blots were quantified using the software Image J. Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test with significance annotated comparing what was detected in the supernatant of each strain to the wild type, and what was detected in the pellet of each strain to the wild type (*$P<0.05$, **$P<0.01$ ***$P<0.001$).

A)

B)

C)

Figure 5.5.4 GLuc expression was assessed in *Δgtr1, Δgtr2, Δvam6, Δatg1* and *Δtco89* strains transformed with either an empty plasmid (marked +e) or with a vector re-expressing the deleted gene. A) Quantified Gluc expression relative to the wild type, of *Δvam6* transformed with an empty plasmid, and re-expression in the deletion stain and wild type (Western blot seen in Figure 5.5.1-3). This is also shown in B) with *Δatg1*, C) with *Δtco89*. Cells were grown overnight in selective media and re-inoculated in YPD at the cell count 1×10^6 and grown to the cell count 1×10^7. Protein was extracted from the pellet using the yeast whole cell quantitative method (von der Haar, 2007) (n=3). Expression of GLuc and actin in the western blots were quantified using the software Image J. Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test with significance annotated comparing what was detected in the supernatant of each strain to the wild type, and what was detected in the pellet of each strain to the wild type (*P<0.05, **P<0.01 ***P<0.001).

As observed in the wild type, *Δgtr1, Δgtr2, Δvam6, Δatg1* and *Δtco89* displayed less GLuc in the supernatant than what was detected in the pellet (Figure 5.5.3A). Deleting the EGO genes *GTR1* and *GTR2* led to a significant loss in secreted GLuc, with an increase in GLuc detected in the pellet of *Δgtr1* (P<0.01) but a loss to GLuc expression in the pellet of *Δgtr2.* Despite changes observed in *Δgtr1* and *Δgtr2* cells, there was no statistically significant alteration to GLuc production in cells lacking the GEF Vam6 in comparison to the wild type. Findings had shown that *Δtco89,* a component of the TORC1 complex, had similar levels of expression of GLuc detected in the supernatant and pellet compared to the wild type. However, deletion of the autophagy gene ATG1 had a negative effect on rP production as we observed a loss of GLuc expression in the pellet in comparison to the wild type (Figure 5.5.3A).

When comparing GLuc expression in the wild type to the Gtr1 deletion strains (Figure 5.5.3B) we discovered that overexpressing the *GTR1* in *Δgtr1* resulted in some rescue to the GLuc rP production observed in *Δgtr1*, however with overexpression of *GTR1* in the wild type we found a significant increase in total GLuc production observed in both the supernatant and pellet. Similarly, some rescue in GLuc expression was observed in Δgtr2 with *GTR2* overexpression to wild type levels, with a further increase in rP expression in the supernatant when overexpressed in the wild type (Figure 5.5.3C). Interestingly, it is only when *Δgtr1* and *Δgtr2* were overexpressed in the wild type that a rescue in the ratio of GLuc detected in the supernatant and pellet was observed as expected yet this was not seen when restoring *GTR1* and *GTR2* levels back into *Δgtr1* and *Δgtr2* respectively. Despite the effects on rP production observed in *GTR1* and *GTR2* strains in comparison to the wild type, alterations to VAM6 caused no changes to GLuc expression observed in the pellet and supernatant (Figure 5.5.4A).

The overexpression of *ATG1* in the *Δatg1* deletion strain caused an increase in the expression of secreted GLuc although this was still not restored to wild type levels, however GLuc detected in the pellet was restored to wild type levels when *ATG1* is overexpressed in *Δatg1* (Figure 5.5.4B). However, when overexpressing *ATG1* in the wild type there was an unexpected decrease in GLuc detected in the supernatant and pellet in comparison to the wild type with normal levels of *ATG1*. *TCO89* overexpression in *Δtco89* appeared to reduce expression and secretion of GLuc compared to the *Δtco89* which had little effect on GLuc expression detected compared to wild type. The percentage of GLuc that was secreted was found to be lower with *TCO89* overexpressed in a wild type background (Figure 5.5.4C).

The deletion of EGO complex genes *Δgtr1* and *Δgtr2* caused a reduction in secretion of GLuc yet not in Δvam6 (Figure 5.5.3A). Our data also suggests that this effect could be rescued when the corresponding gene was re-expressed (Figure 5.5.3B,C) yet this was not the case with *ATG1* overexpression. We found the most significant effects in *Δgtr1* cells where overexpression of GTR1

which had a significant increase in secretion compared to the wild type. Overexpression of TCO89 surprisingly had a negative effect on GLuc production compared to the wild type in both Δtco89+TCO89 and WT+TCO89 (Figure 5.5.4H).

5.2.3.3 Growth analysis of overexpression in genes in the TOR pathway

We wished to determine whether changes in growth rate associated with overexpression of genes within the TOR signalling pathway could account for variation in Gluc expression. The growth rate of strains deleted for *VAM6, TCO89, GTR1, GTR2, and ATG1* transformed with either an empty vector or vector expressing the deleted gene was compared to that of wild type. An example of the growth curve data is presented in Fig 5.8A and doubling times of *VAM6, ATG1, GTR1, GTR2 and TCO89* all strains in Fig 5.6.B

A)

B)
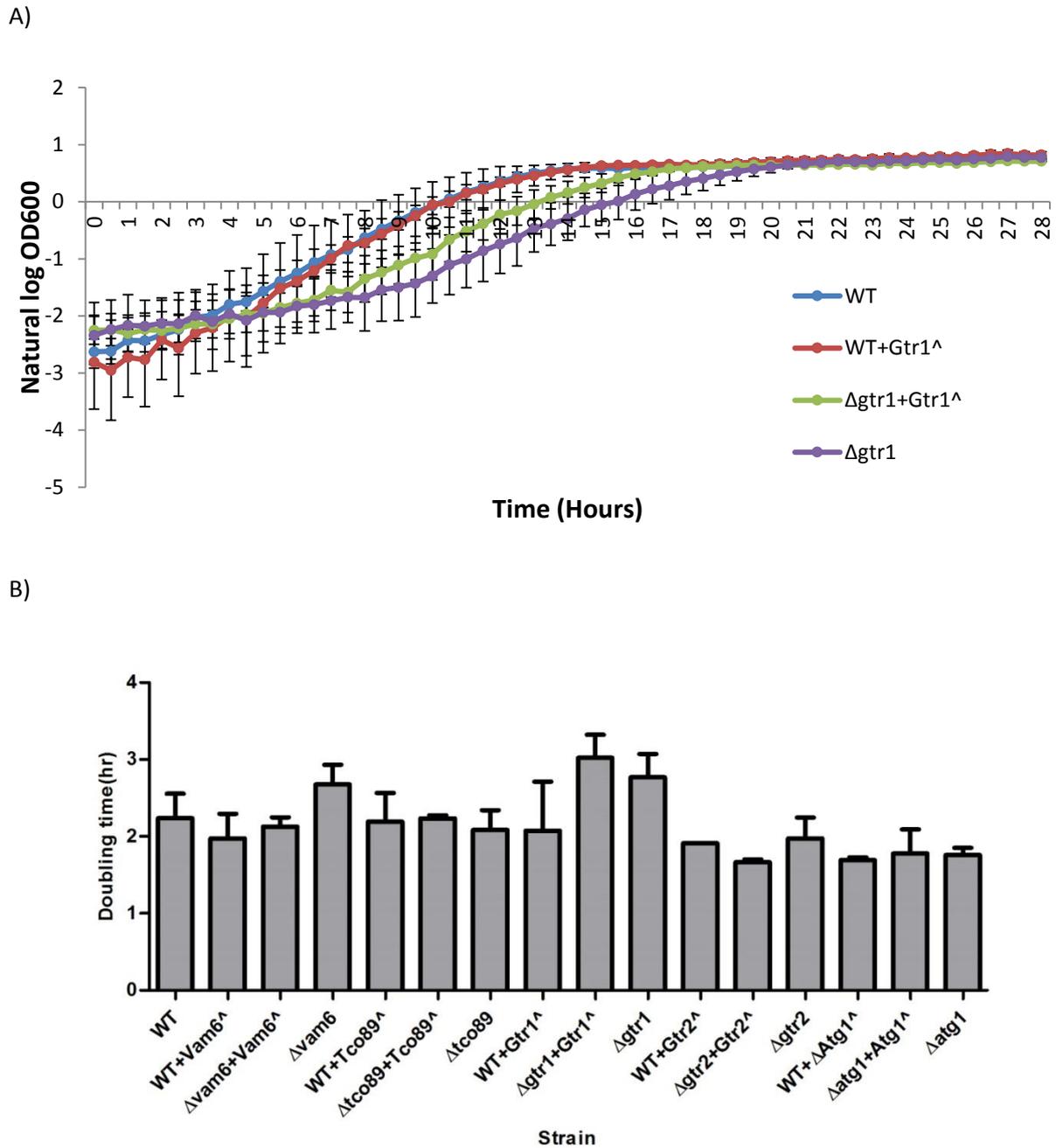
Figure 5.6 Growth of Δvam6, Δtco89, Δgtr1, Δgtr2 and Δatg1 with VAM6, TCO89, GTR2, and ATG1 gene overexpression. A) Growth curves of the wild type, Δgtr1, Δgtr1 with GTR1 overexpression and wild type with GTR1 overexpression. B) Doubling times were calculated in all selected TOR strains. Cells were grown overnight in minimal media before re-inoculated into YPD to an OD_{600} of 0.3 (n=3). Errors bars represent standard deviation. Statistical analysis was performed using an ANOVA test with significance annotated comparing each strain to wild type (*P<0.05, **P<0.01 ***P<0.001).

The data from our growth analysis in figure 5.6B shows that wild type cells exhibit a doubling time of approximately 2 hours. We observed a longer lag phase in *Δgtr1* compared to the wild type (Figure 5.6A), however once in log phase there was no significant change to doubling time displayed in figure 5.6b when the doubling time was quantified. When comparing the growth of all deletion strains and when their gene is overexpressed has caused no change to growth compared to the wild type concluding we can rule out any changes to growth effecting changes to GLuc expression seen in figure 5.5.

5.2.2.4 Investigating growth analysis in GTR1 with rapamycin treatment

Our results suggested that manipulation of the EGO complex, and in particular Gtr1, leads to a significant effect upon GLuc expression. We wished to determine whether TORC1 signalling was compromised within cells lacking or overexpressing Gtr1 by testing their sensitivity to rapamycin (Figure 5.7).
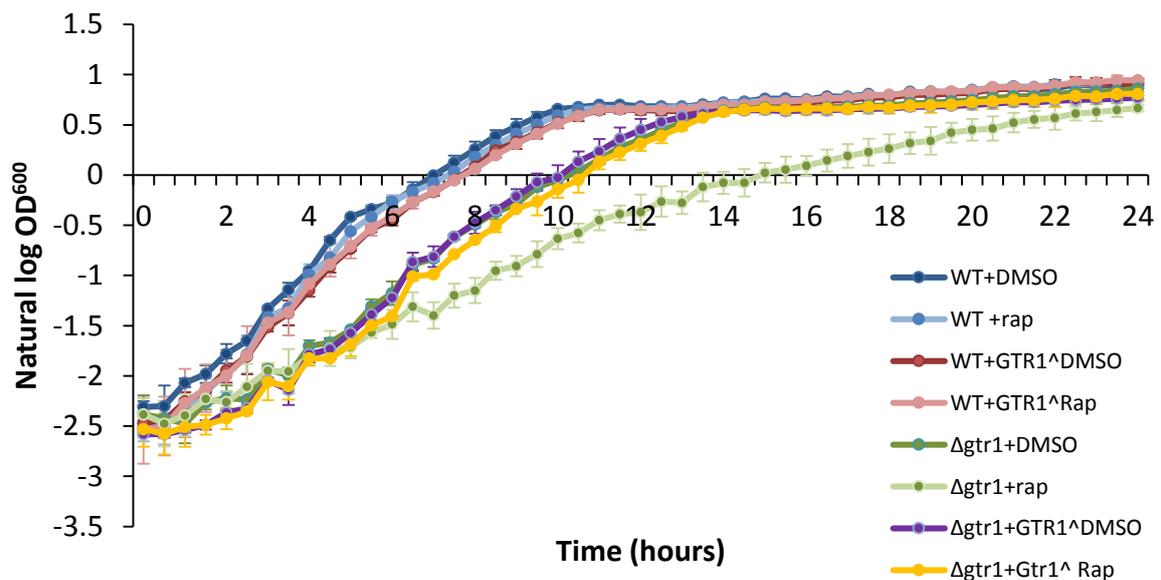
Figure 5.7 Growth with rapamycin of GTR1 deletion and overexpression of GTR1 in the deletion strain and wild type. Cells were grown overnight in YPD media containing either DMSO as a control or 3 ng/ml rapamycin before being re-inoculated in YPD to an OD_{600} of 0.1 (n=3) where either rapamycin or DMSO were added again. Error bars represent standard deviation.

Compared to growth with DMSO, rapamycin treatment had no effect on growth in the wild type and WT+*GTR1^.* It was observed that only when *GTR1* was deleted that the cells become sensitive to the effects of rapamycin, yet upon re-expression, the effects were reversed, although not fully to growth of the wild type (Figure 5.7). This test showed that deleting *GTR1* made cells more sensitive to inhibition of TORC1 at this concentration of drug. Due to the additive effect of rapamycin to *Δgtr1*, this suggests that the TORC1 pathway is functional in cells lacking GTR1 and may imply that rP levels are altered in a manner that is independent of TOR signalling.

5.2.4 Effects of deletion of genes involved in TOR signalling upon Killer toxin secretion

We employed the killer toxin assay as another method to assess rP production in strains lacking the genes *GTR1, GTR2, VAM6, TCO89 and ATG1*. A plasmid containing the killer toxin plasmid was transformed into strains lacking the genes *GTR1, GTR2, VAM6, TCO89* and *ATG1* where these strains were grown into log phase and were spotted in a dilution series on to a lawn of yeast cells which are known to be sensitive to the toxin. As a result a zone of clearance is produced which represents the level of protein secretion and which can be measured (see figure 5.8 below).

Strain   Control
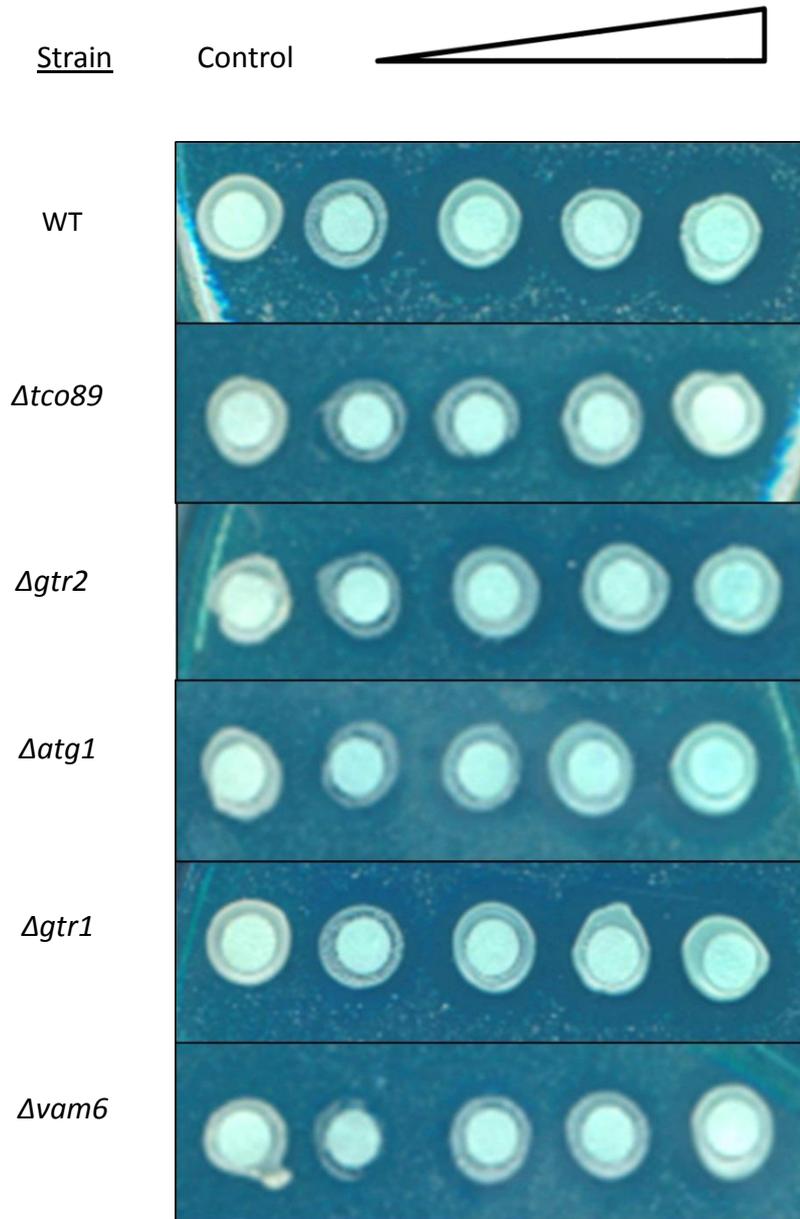
Figure 5.8.1 Example of clearance zones created in killer toxin assay in the wild type, *Δgtr1, Δgtr2, Δtco89, Δatg1* and *Δvam6*. Left spot contains strain without the killer toxin plasmid, spots to follow have increasing cell count. Killer strains were grown overnight in selective media and re-inoculated the following morning to a cell OD_{600} of 0.2 in selective media and grown to the cell count $1x10^7$cells/ml.5-fold, 2.5- fold and 2-fold dilutions were performed and cells spotted onto plates with the final spot equal to a cell count of count $1.3x10^6$. (n=3).Plates where left to grow at 30°C for 2-3 days. Image J was used to quantify kill zone and image contrast enhanced by 0.1%. Statistical analysis was performed using an ANOVA test with significance annotated comparing each strain to wild type (*P<0.05, **P<0.01 ***P<0.001).Errors bars represent standard deviation. For more details on measuring the clearance zone, see chapter 3, figure 3.10.
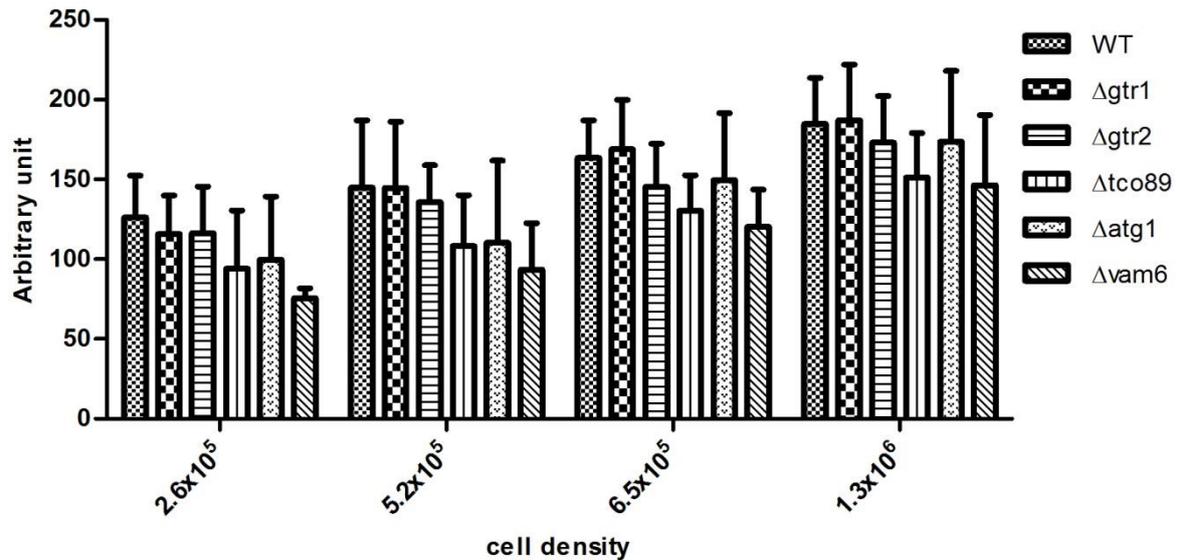
Figure 5.8.2 Quantification of the clearance zone from killer toxin in the wild type, *Δgtr1, Δgtr2, Δtco89, Δatg1* and *Δvam6.* Clearance zone was measured using the software Image J. Killer strains were grown overnight in selective media and re-inoculated the following morning to a cell OD_{600} of 0.2 in selective media and grown to the cell count 1×10^{7}cells/ml.5-fold, 2.5- fold and 2-fold dilutions were performed and cells spotted onto plates with the final spot equal to a cell count of count 1.3×10^{6}. (n=3).Plates where left to grow at 30°C for 2-3 days. Statistical analysis was performed using an ANOVA test with significance annotated comparing each strain to wild type (*$P<0.05$, **$P<0.01$ ***$P<0.001$).Errors bars represent standard deviation. For more details on measuring the clearance zone, see chapter 3, figure 3.10.

This data presented shows a steady increase in the clearance zone with increasing cell density in all strains tested (Figure 5.8.2). At all cell densities, we detected no significant change in secretion of the toxin in the strains *Δgtr1, Δgtr2, Δtco89, Δatg1* and *Δvam6* compared to that of wild type (Figure 5.8.2). These data show that *Δgtr1, Δgtr2, Δtco89, Δatg1* and *Δvam6* had similar secretion to the wild type suggesting deletion of these genes had no effect on secretion of the toxin, as opposed to secretion of the GLuc protein (Figure 5.5.3A).

We also assessed whether the secretion of killer toxin itself had an effect upon growth in the strains assessed. The doubling times were therefore calculated in wild type, *Δtco89*, *Δvam6*, *Δgtr1, Δgtr2* and *Δatg1* cells transformed with either a control empty vector or the killer toxin plasmid (Figure 5.9).

A)
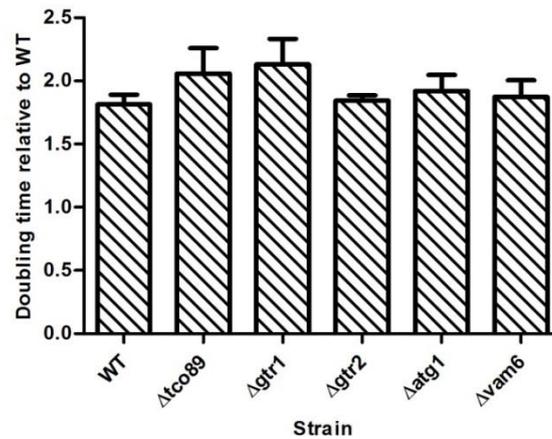
B)
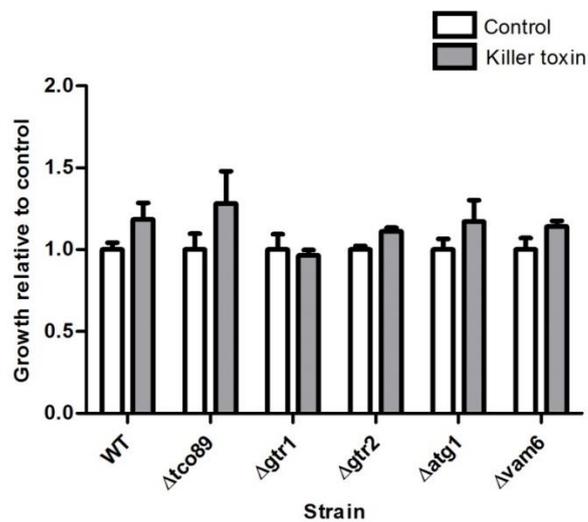
Figure 5.9 Effects of Killer Toxin expression on growth of selected strains. a) Doubling time of strains deleted for *TCO89, GTR1, GTR2, ATG1* and *VAM6* without the killer toxin. Statistical analysis was performed using ANOVA test with significance annotated comparing each strain to wild type b) Growth of strains deleted for *TCO89, GTR1, GTR2, ATG1* and *VAM6* expressing killer toxin relative to strains without (n=2). Statistical analysis was performed using an ANOVA test with significance annotated comparing growth of each strain with the control plasmid to the killer toxin plasmid. Cells were grown overnight in selective media before re-inoculated to an OD_{600} of 0.3. (*$P<0.05$, **$P<0.01$ ***$P<0.001$).Errors bars represent standard deviation.

The expression of killer toxin appeared to have no effect upon the doubling time of wild type, *Δgtr1, Δgtr2, Δtco89, Δatg1* or *Δvam6* cells.

5.3 Discussion

The drug rapamycin, inhibits TORC1 signalling and is known to cause a decrease in protein synthesis and ribosomal biogenesis, thus mimicking responses to starvation (Dubouloz *et al.*, 2005). Rapamycin treatment reduces cell size in both lower and higher eukaryotes and causes reduced antibody production in CHO cells (Fingar *et al.*, 2002; Courtes *et al.*, 2013). Due to our interest in rP production, we investigated GLuc expression on wild type cells with rapamycin treatment in yeast (Figure 5.2.1). A loss in expression of GLuc was observed in the supernatant when yeast cells were treated with a level of rapamycin that did not affect doubling time (Figure 5.2.2). This suggested TORC1 signalling may be manipulated to change rP production in yeast cells. To identify individual genes that may be useful targets for the development of enhanced rP production we screened a wide range of upstream and downstream regulators within the TORC1 network for their ability to influence the synthesis and secretion of a model protein, Gluc.

5.3.1 *Gaussia* luciferase expression screen identifying genes in the TORC1 pathway that impact upon recombinant protein production

Strains from the yeast knockout collection involved in variety of processes which hinder or facilitate protein synthesis through the TORC1 pathway were utilised in a screen to identify new targets that may alter rP production (Figure 5.3). By deleting the gene coding for the protein Tip41, which operates in the complex downstream of TORC1, we observed an increase in GLuc protein detected in the pellet suggesting there was an increase in protein synthesis but no change in secretion (Figure 5.3.3B). The increase in GLuc expression in *Δtip41* could be explained by the fact that this part of the pathway is inhibited by the TORC1 complex during cell growth and protein production (Rohde *et al.*, 2008), yet we found deletion of *SIT4* decreased GLuc production. Tip41 signals downstream to genes controlling the retrograde response (*RTG1/3*) and the nitrogen discrimination pathway (*GLN3*) which were found to decrease GLuc expression when deleted (Figure 5.3.3D), suggesting that deleting *TIP41* improved GLuc expression due to another role other than downstream signalling in TORC1 (Smets *et al.*, 2010). For cell growth and protein synthesis, the TOR pathway would repress transcription of starvation-specific genes during favourable nutrient conditions by sequestering Msn2, Msn4, Gln3, Rtg1, Rtg3 in the cytoplasm. This suggests that deleting these stress TF should be favourable for rP production as was seen with *Δgis1* (Figure 5.3.4A), yet we found a loss of GLuc production in *Δmsn4, Δgln3 Δgcn4, Δrtg1* and *Δrtg3* (Figure 5.3.4A, 5.3.5C, 5.3.3D) (Marion *et al.*, 2004). The deletion of *Δgln3, Δgcn4, Δrtg1* and *Δrtg3*, leaves the cells incapable of adapting as it cannot detect glutamine starvation, however this experiment was performed during log phase where there were still plenty of nutrients

in the media, nevertheless there was a loss in GLuc production when these genes were deleted compared to the wild type. In a similar way, it was surprising that deleting autophagy genes would have a negative impact on rP production. However it has been noted that some level of autophagy is still important during log phase, preventing the build-up of protein aggregates (Mizushima *et al.*, 2008).

As expected, important genes involved in ribosomal proteins and biogenesis (*RPS6A, RPS6B* and *SFP1*) when deleted caused a loss in GLuc production as this will deter protein synthesis (Figure 5.5.5A), yet removing the repressor of translation *CRF1* also had a negative impact on GLuc production (Figure 5.3.5B). TOR1/2 in TORC1 is necessary for rP production, when deleting *TOR1* there was no change to GLuc production as the gene *TOR2* would still be available as a component of TORC1 (Figure 5.3.4D). Another component of the TORC1 when deleted had shown no significant change to GLuc production, Δtco89, yet a new model suggests that Tco89 may bind to Gtr1 in TORC1 activation during nutrient sensing (Sekiguchi *et al.*, 2014).

When deleting genes involved in stress response such as osmoregulation (Δpkh2, Δsko1 and Δhog1) (Figure 5.3.3), nutrient starvation such as nitrogen (*Δnrp1*)(Figure 5.3.5D) and amino acids (*Δego1*)(Figure 5.3.4B) we found a decrease in GLuc produced as when the cell is being pushed to produce rP production this puts mores stress on the cell. However, not all genes involved in amino acid sensing when deleted caused a loss in GLuc production. The EGO complex which comprises of Ego1 and Ego3, anchors the heterodimer Gtr1 and Gtr2 to the vacuole where this complex senses amino acid levels. Deletion of any of these components causes low TORC1 activity inhibiting protein synthesis and cell growth, essentially triggering starvation responses, even in nutrient rich media (Zhang *et al.*, 2012). It would have been more feasible for a loss of GLuc production in *Δego3* than *Δego1* as Ego3 functions as a homodimer, mediating the interaction between Ego1 and the GTR proteins (Figure 5.4.3B) (Zhang *et al.*, 2012). However, more recent literature suggests a model where deleting the protein Ego1 may cause a loss of interaction between Ego3 and the GTR proteins, and that it may be the Ego1-Ego3 complex acting as a GEF to the GTR proteins (Sekiguchi *et al.*, 2014). This contradicts previous literature which suggests that Vam6 is the protein which regulates the GTP-GDP exchange on both GTR proteins needed for signalling to TORC1 (Valbuena *et al.*, 2012). Additionally, it is the GTR proteins which interact directly with TORC1 yet there was no significant change to GLuc production when *GTR1* or *GTR2* were removed.

We discovered that 15 of the 30 genes investigated, which were grouped together according to their role in TORC1, caused a loss in GLuc expression when deleted reiterating the importance of TORC1 signalling in rP production. This is particularly interesting as strains expressing low GLuc are linked to

stress response and protein synthesis, vital for the correct homeostasis of the cell and rP production. Overexpression of GLuc, a heterologous protein at high levels, would be connected to different stresses as this may limit other cellular processes by competing for substrates (Mattanovich *et al.*, 2004).

5.3.2 The effects of overexpression of *VAM6, ATG1, GTR1, GTR2* and *TCO89* upon rP production

For further analyses of the selected genes *VAM6, ATG1, GTR1, GTR2 and TCO89*, we used an expression vector to re-express in the appropriate knockout strains as well as to overexpress in a wild type background. We observed no significant change to growth when comparing the doubling time of *Δvam6, Δatg1, Δgtr1, Δgtr2* and *Δtco89* to the wild type. A similar result was observed when these genes were overexpressed in the corresponding deletion strain, (Figure 5.6). This data implies that changes to growth rate were not responsible for differences in GLuc expression or secretion.

5.3.2.1 Effects of Δatg1 on recombinant protein production

A great deal of research has been undertaken to understand the process of autophagy. TORC1 negatively regulates autophagy by the hyperphosphorylation of Atg13, thus preventing the formation of the Atg13-Atg1 complex. Autophagy is also regulated by the nutrient sensing pathways PKA and the energy sensing AMPK pathway (Scott *et al.*, 2007). When investigating the role of *ATG1* in GLuc expression we discovered that its deletion led to reduced levels of GLuc in both the supernatant and pellet suggesting it affects overall protein synthesis (Figure 5.5.4B). As protein extracts were taken during log phase, when cells have sufficient available nutrients in their growth media, it was surprising to find Δatg1 had any effect. However, it has been noted that autophagy is utilised during active growth as part of the protein homeostasis system to prevent a build-up of protein aggregates. Therefore a basal level of autophagy during growth is important for maintaining normal cellular homeostasis (Mizushima *et al.*, 2008). Misfolded protein accumulation in the ER can send stress signals that induce autophagy as an alternative pathway for ER quality control. This is also verified by the induction of autophagy with rapamycin treatment where the cells were found to be more resistant to ER stress (Yorimitsu and Klionsky, 2007). Although our data shows that Atg1 expression impacts upon rP production, we were only able to partially rescue the reduction in Gluc expression observed in *ATG1* null cells when re-expressing the gene compared to the wild type (Figure 5.5.4B). This shows that *ATG1* is important in rP production yet we observed a loss to GLuc when overexpressed in the wild type.

However, in this study as we did not determine protein levels. A future validation of the results here would be to use an antibody to investigate *ATG1* overexpression as it did not appear as if the mRNA levels were any higher than the control in the wild type suggesting this plasmid may not be working (Figure 5.3C). Yet, we found that overexpression caused a reduction to GLuc detected in the supernatant and pellet in the wild type suggesting the plasmid is working and that subtle changes in the amounts may have a detrimental effect on protein synthesis. High *ATG1* levels may increase autophagy in the cell to a level too high reducing protein synthesis (also reported in the literature); autophagy has a negative feedback on S6 (Sch9 in yeast) to reduce growth of cells in an environment lacking nutrients or under stress (Scott *et al.*, 2007; Lee *et al.*, 2007). Discrepancies between mRNA levels and the effects of the plasmid on rP production may reflect how mRNA levels don't always correlate to expected protein levels as many biological factors can influence this effecting translation rates. Conversely, even if translation rates were all the same, this does not take into any differences with, or account for changes in protein stability (Maier *et al.*, 2009).

5.3.2.2 Effects of Δtco89 on recombinant protein production

Tco89 has been identified as part of the TORC1 complex and found to localise to the vacuole as well as Kog1 (another TORC1 complex subunit), yet was originally discovered due to its role in cell integrity (Reinke *et al.*, 2004; Binda *et al.*, 2009). It was suggested by Sekiguchi et al. (2014) that the C-terminal of the GTPase Gtr1 (part of the EGO complex) interacts with Tco89 when Gtr1 is GTP bound (Sekiguchi *et al.*, 2014) having a direct effect on amino acid sensing. Upon deletion of TCO89 we found no significant change to GLuc expression (Figure 5.5.4C). However, overexpression led to a loss in Gluc expression in the supernatant and pellet when overexpressed in the wild type suggesting this causes a negative effect on protein synthesis. Little is known about the role(s) of *TCO89* in the cell and understanding the mechanism behind this phenotype; therefore it is not known if Tco89 has any inhibitory effects on processes in the cell that may be important for protein synthesis. Being directly part of TORC1, any changes may affect downstream signalling to proteins involved in protein synthesis such as Sch9 or by perturbing the balance of stress signalling important in growth control (C De Virgilio and Loewith, 2006b). Overexpression of *TCO89* causes increased protein-protein interactions between Tco89 and other known associating proteins in TORC1 and perhaps other unknown interactions, inhibiting protein functions linked to downstream protein synthesis. However, it was recently suggested that within the EGO complex, the component Gtr1 may directly bind to TCO89, increasing TORC1 signalling through nutrient sensing. However, overexpression of protein can overload the cell becoming detrimental by increasing a number of intracellular stresses such as protein aggregation although the plasmid used for this study had a low copy number (Naidoo, 2009). Overexpression of

TCO89 in the wild type caused a loss in GLuc in the supernatant impacting the secretory pathway of the cell. This could be due to a build-up of proteins aggregating as the quality control was not sufficient for successful increased secretion which would lead to ER stress (Yorimitsu *et al.*, 2006). There may have been an increase in ER stress markers such as phospho-PERK or Bip which could be investigated in future work (Vannuvel *et al.*, 2013).

In conclusion the data presented here provides evidence that Tco89 may have a negative effect on recombinant protein production although at this time we do not understand the mechanisms behind this due to the limited knowledge on its role in TOR signalling.

5.3.2.3 The effects of deleting EGO complex components on recombinant protein production
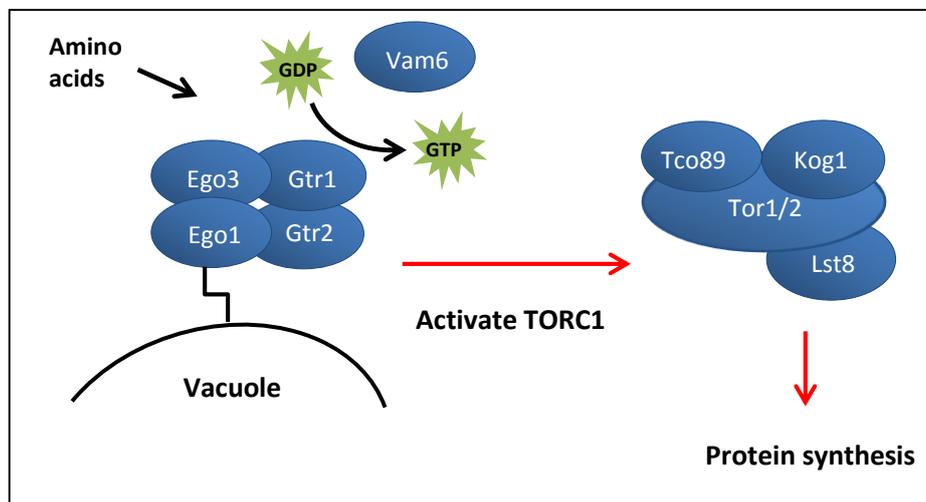
Figure 5.10 The EGO complex in yeast and mammalian systems needed for amino acid sensing in the TORC1 pathway. The complex in yeast consists of the heterodimer Gtr1 and Gtr2 binding to Gse1/Ego3 and Gse2/Ego1. Figure taken from Kogan et al., (2010).

The EGO complex contains Ego1 and Ego3 involved in anchoring the complex to the vacuole, and Gtr1 and Gtr2 which are known to activate TORC1 via a still elusive mechanism (Binda et al., 2009) (Figure 1.8). The human homologue of Gtr1 is RagA and RagB, and binds to the Gtr2 homologue of RagC and RagD to form a heterodimer. This regulates TORC1 protein kinase activity through amino acid signalling by controlling its intracellular localisation (Sekiguchi *et al.*, 2014). Both Gtr1 and Gtr2 are involved in the regulation of Gap1 amino acid permease which is controlled by nitrogen source (Binda *et al.*, 2009).

If cells can't sense nutrients due to mutations in the EGO complex this has a knock on effect where cell growth slows, entering quiescence and starvation state where transcription factors such as Gcn4 activate transcription of genes needed for amino acid biosynthesis; this is due to TOR depletion or rapamycin treatment (Dubouloz *et al.*, 2005; Smets *et al.*, 2010). Partial inactivation of TORC1 also leads to loss of sorting Gap1 from the late endosome to the plasma membrane leading to loss of amino acids and nitrogen starvation (Inoki *et al.*, 2005). Deletion of any of the EGO genes leading to loss of rP production suggests that the cells were starving even when grown in nutrient rich media leading to down regulation of protein synthesis through the TORC1 pathway due to changes in nutrient signalling. Studying the metabolomics of these EGO strains would tell us more about how the cell is surviving when *amino acid sensing is lost in GTR1/GTR2* and what nutrient source is being used.

Although the transition from quiescence back to proliferation from loss of TORC1 signalling is poorly understood, it is believe that EGO complex ensures proper exit from rapamycin-induced growth arrest by regulating microautophagy (in response to glutamine levels) to counter-balance the macroautophagy-mediated membrane influx toward the vacuolar membrane (Dubouloz *et al.*, 2005; Li *et al.*, 2012). Therefore, as overproduction of the EGO complex is known to induce microautophagy and in this study we found that overproduction of *GTR1* in the wild type caused an increase in GLuc production, suggesting that by increasing microautophagy through the EGO complex this improved rP production enables the cell to direct uptake and degradation of the vacuolar boundary membrane (shrinking the vacuole), increasing nutrient concentration and making these available for cellular processes (Uttenweiler and Mayer, 2008; Li *et al.*, 2012) (Figure 5.5.3B). Overexpression of the autophagy gene *ATG1* did not cause the same effect which could be due to the importance in the balance between autophagy and TOR signalling is important in cell aging and fitness (Hands *et al.*, 2009) (Figure 5.4G).

Removal of *GTR1* caused a loss in GLuc in the supernatant with an increase of GLuc in the pellet suggesting this mutation impacts negatively on secretion of the rP (Figure 5.5.3A). This in in contrast to deletion of *GTR2* which impacted negatively on total recombinant protein production as there was a loss of GLuc detected in both the supernatant and pellet. With overexpression of *GTR1* in its deletion strain, we found some rescue of GLuc expression to wild type levels in the pellet. However, when overexpressed in the wild type, this caused an increase in GLuc production, roughly 3 times what was detected without overexpression of *GTR1* (Figure 5.5.3B). This data suggests that *GTR1* impacts upon rP production with enhanced production when *GTR1* is overexpressed. We observed the highest mRNA levels in the *GTR1* overexpression plasmid compared to other plasmids which may add to the explanation for the large increase in GLuc production when *GTR1* was overexpressed in the wild type.

However, as the protein levels of Gtr1 were not confirmed, it would be beneficial to check Gtr1 levels when using the overexpression plasmid in the wild type and measure GLuc production to confirm this is due to *GTR1* over-expression.

Results from investigating GLuc production in GTR2 strains show some rescue of GLuc expression with *GTR2* overexpressed in *Δgtr2* to wild type levels detected in the supernatant yet with full rescue of GLuc detected in the pellet (Figure 5.5.3C). Although the loss of *GTR2* has a negative impact on rP production, we also found that when overexpressing this gene in the wild type, this too has negative implications suggesting there is a delicate balance between overabundance of Gtr2 and loss of its function by removal.

This difference between the heterodimers is interesting, signifying differences in other roles which are currently not well understood (Sekiguchi *et al.*, 2014). A new model as to how the EGO complex interacts with TORC1 has been proposed where Tco89 binds to Gtr1 (GTP bound) and Kog1 bins to Gtr2 in order to signal to TORC1 (Sekiguchi *et al.*, 2014). It was also discovered that Gtr1 can also form a homodimer as well as a heterodimer with Gtr2, which may explain why overexpression of *GTR1* has a stronger effect on rP production (Valbuena *et al.*, 2012). The interactions of Gtr1 with nuclear and cytoplasmic proteins Rpc19p (RNA polymerase subunit), Nop8 (nucleolar protein) and Yrb2 (controlling nuclear protein export) as well as the deletion of Gtr1 reducing RNA pol I and III activity suggests a role for Gtr1 in RNA synthesis and ribosome maturation effecting rP production (Wang *et al.*, 2005; Todaka *et al.*, 2005; Wang *et al.*, 2009). These are examples of roles outside of amino acid signalling that might influence rP production through the overexpression of GTR1. If a polysome profile of the GTR1 strains were tested in the future, this could give us more information on how changes to GTR1 expression levels are affecting GLuc production and if there are reduced polysomes. Sekiguchi *et al* have discovered that both proteins interact with the chromatin remodelling factor INO80 complex. *GTR1* is also believed to interact with *INO80* and Gtr2 interacts with Rvb1 and Rvb2 (also involved in the INO80 complex) (Sekiguchi *et al.*, 2008).

In the initial screen we found that *Δego1* caused a significant decrease to GLuc production, however this was not repeated or taken any further as we wanted to investigate the GTR proteins which directly activate TORC1 (Figure 5.3.3B). As we know the EGO complex is important in activating TORC1, another experiment would be to repeat this test alongside determining any changes to GLuc production with EGO1 overexpression in the deletion strain and the wild type.

When quantifying the western blot of GLuc expression the Vam6 strains through statistical analyses we found that Vam6 strains caused no change to GLuc expression suggesting this deletion caused no

change to amino acid sensing through the EGO complex (Figure 5.5.4A). Valbuena et al (2012) reported that in *S.pombe*, the Δvam6 orthologue still had partial response to amino acids, indicating another unknown protein involved in a similar role to Vam6 or there was a problem with the deletion strain. It has been suggested that Ego1 and Ego3 may play a role in TORC1 activation as the regulatory complex which has structural conservation with the Ego proteins in mammalian cells has GEF activity towards the Rag proteins (equivalent of GTR proteins) (Zhang *et al.*, 2012). Vam6 mutants present with fragmented vacuolar morphology which can be seen using GFP-Vam6 so it would be worth checking that this deletion strain has this characteristic to confirm this gene was deleted correctly. Vam6 is paralogous to Vam2 but has not been linked as a controller of guanine nucleotide exchanger factor for GTR proteins (Ostrowicz *et al.*, 2010). When overexpressing *VAM6* in *Δvam6* and the wild type there was no change to GLuc which is expected as deleting VAM6 showed no change, however when measuring mRNA levels in the wild type with the overexpression plasmid we found levels to be similar to the wild type suggesting the plasmid may have not been working (Figure 5.4A). As mentioned before when discussing ATG1 overexpression, this may be because mRNA levels aren't depicting protein levels accurately.

5.3.3 TOR inhibition in Gtr1 strains

As *GTR1* overexpression exhibited the largest increase in rP production when overexpressed in the wild type we had chosen to test the effects of rapamycin on the growth of these strains. 3 ng/ml of rapamycin was added to YPD media to the wild type and all Gtr1 strains (Figure 5.7). This concentration of rapamycin had little or no effect on growth of almost all strains except *Δgtr1* which showed sensitivity to the drug. When treating cells with rapamycin, transcription factors are triggered for nuclear entry which stimulates gene expression of products required for the synthesis of nutrient storage reserves, for the uptake and assimilation of suboptimal nitrogen sources, synthesis of amino acids and cell survival under stress conditions (Claudio De Virgilio and Loewith, 2006). We found that *Δgtr1* had repressed growth implicating that it is involved in curating some of these processes. However, *Δgtr1* sensitivity to rapamycin suggests TORC1 signalling in not completely inhibited by the removal of the amino acid sensing gene which may indicate that GTR1 plays other roles in the cell parallel to TORC1 signalling. When *GTR1* was overexpressed in *Δgtr1*, this decrease in doubling time was rescued (Figure 5.7).

The data presented in this chapter was not consistent throughout. The loss of genes GTR1, GTR2 and ATG1 in the screen showed no change in GLuc production (Figure 5.3), in contrast when this was

repeated with the selected strains (Figure 5.5); there was a loss in GLuc production in triplicate analyses. However, due to the lack of a loading control in the screen performed, the data seen may not be a true representation even though all samples were diluted to the same cell count. The selected strains from the screen were then tested using an alternative method which is believed to be a more quantitative protocol of releasing the protein from cells (through chemical disruption rather than mechanical), however the supernatant was treated the same way yet results were varied. When using this second method, cells where grown for longer (to 1×10^7 cells) as a much smaller number of samples were tested making the experiment more manageable compared to the screen where all cells were grown for the same amount of time which may cause slight variation to phase of growth, perhaps to early log phase.

There has also been evidence that there are some knockouts possessing background mutation(s) or perhaps epigenetics which may explain why we don't see full rescue of GLuc production to what was detected in the wild type in *Δgtr1, Δgtr2* and *Δatg1* when their respective genes are overexpressed (Figure 5.5.3B,C and 5.5.4B). As TORC1 signalling is involved in a number of processes such as protein synthesis, responses to stress and nutrient sensing, as such trying to pull apart this complex is difficult as it also cross talks with a number of other sensor pathways. However, there is much work into investigating the GTR proteins in the EGO complex, and the GTR1 data from this chapter is more reproducible.

5.3.4 Killer Toxin secretion in *Δgtr1, Δgtr2, Δvam6, Δatg1* and *Δtco89*

To test the recombinant protein production of these strains with another protein other than GLuc, we used the killer toxin assay by measuring the secretion of the toxin by the size of the kill zone over a lawn of sensitive cells (Figure 5.8.2). When comparing secretion by the cells with the highest cell count we found no statistical difference to secretion, which was not in agreement with the secretion of GLuc reported in figures 5.5.3A, where *Δgtr1, Δgtr2* and *Δatg1* show a decrease. However, due to these proteins containing different signal sequences, they should not be directly compared. The doubling times of these strains with and without the killer toxin assay exhibited no change to growth (Figure 5.9). Generally we did not find these deletions causing the same effects on toxin secretion as what was found with GLuc secretion.

5.3.5 Conclusion

In this chapter we performed a GLuc expression screen to select for new targets within the TORC1 pathways which alter rP production. Due to some discrepancies in data replicates, we cannot draw absolute conclusions in all deletions, however the data does imply the EGO complex, specifically *GTR1* and *GTR2,* when using the GLuc expression plasmid with the alpha factor signal sequence are important determinants of GLuc expression. Recent data points towards emerging roles of GTR1 and GTR2 in chromatin regulation and ribosomal regulation connecting EGO nutrient signalling to epigenetics, yet only overexpression of GTR1 in the wild type caused an increase in rP production reflecting differences in the role of the GTR proteins. We also found with Δgtr1 that TORC1 signalling can be uncoupled from growth rate when treated with rapamycin suggesting roles in GTR1 independent of TORC1 signalling are causing altered rP production. However, as the expression of GLuc was driven by the alpha factor promoter, this may have an unknown impact on TORC1 signalling. Variation in data means the observed results would need to be further verified, nevertheless, the data does imply and identify certain target genes as being worthy of further investigation as to their influence on rp production.

Chapter 6

Evaluation and Validation of mTOR Targets Identified in Yeast in a Model Mammalian Expression System

6.1 Major constituents of the TOR signalling pathway are conserved from yeast to higher eukaryotes

TOR is a key signalling pathway as described in the introduction chapter to this thesis; therefore it is not surprising that the key components of TOR signalling are well conserved across eukaryotes where the tight control/regulation of growth and nutrient sensing is fundamental for survival. Indeed, the main regulators in the TOR pathway transcend from yeast to higher eukaryotes, although some features are not entirely identical. For example, the protein TCO89, is a component of TORC1 that is only conserved among fungi (Reinke *et al.*, 2004; Sekiguchi *et al.*, 2014). Although yeast are considered one of the lower eukaryotes, *S.cerevisiae* is an important model eukaryotic system for investigations into the functions of TOR, with only one of the two TOR complexes being sensitive to rapamycin (Claudio De Virgilio and Loewith, 2006). Unlike yeast, mammalian cells contain only one gene for TOR, termed mTOR, where the protein from this gene is found in both the TORC1 and TORC2 complex, where activity is blocked by rapamycin which binds to FKBP12 preventing the ability of mTORC1 to phosphorylate downstream substrates (Michnick *et al.*, 1991; Dann and Thomas, 2006). The main components of TOR signalling in yeast and mammalian cells and the relationship/comparison between these are described in Table 6.1 below.
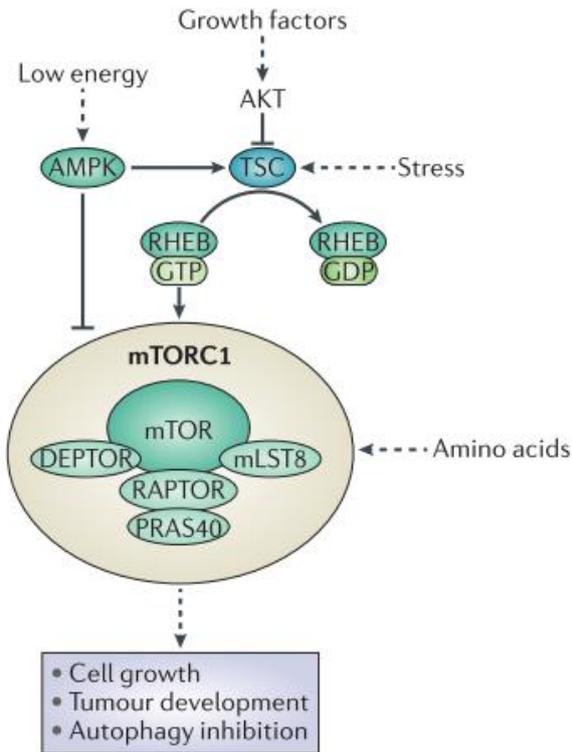
Figure 6.1 A schematic showing the basics of the mTORC1 pathway in mammals. Environmental inputs and signals such as energy availability, growth factors, stress and amino acid availability are sensed and act upstream of TORC1. TORC1 is regulated by amino acids via the regulatory complex containing RAG GTPases which is controlled by GEF activity. AMPK phosphorylates RAPTOR and TSC2 inhibiting mTORC1 signalling. Activation leads to cell growth, tumour development and inhibition of autophagy (Jewell *et al.*, 2013).

Through biochemical purification TOR was found to be associated with a high-molecular weight complex (Loewith *et al.*, 2002) (see mTORC1 figure 6.1). In mammalian cells, mTORC1 contains the protein Raptor that is believed to function as a scaffold coupling mTOR to substrates (regulatory associated protein of mTOR), which was discovered to be important for phosphorylation of S6K and 4EBP1 (involved in regulating protein synthesis by binding and sequestering the translation initiation factor eIF4E) (Inoki *et al.*, 2005; C De Virgilio and Loewith, 2006a; Sancak *et al.*, 2008). The complex also contains the protein mLST8 whose function is not well elucidated but has been implicated in negatively regulating amino acid biosynthesis (Chen and Kaiser, 2003; Wang and Proud, 2011). AMPK (AMP-activated protein kinase) inhibits mTORC1 when cells are energy deprived by phosphorylating raptor (Dunlop and Tee, 2013). This kinase is believed to be a primary physiological sensor of the intracellular AMP/ATP ratio, important for maintaining the energy requirements in the cell (Inoki *et al.*, 2005). In yeast the homologue of AMPK is SNF1, a glucose sensory protein kinase which promotes

GLN3 and the expression of nitrogen-catabolite repression sensitive genes, which allows cells to import and utilise poor nitrogen sources(Bertram *et al.*, 2002; Engelberg *et al.*, 2014).

Tuberous sclerosis (TSC) is a disorder causing benign tumours in a variety of organs, and the TSC1 and TSC2 proteins have been shown to negatively regulate TOR (Inoki *et al.*, 2002; Han *et al.*, 2012). AMPK inhibits mTORC1 through phosphorylation of TSC2. TSC2 forms a complex with TSC1 which negatively regulates TOR signalling as overexpression of TSC1 and TSC2 suppresses S6K and 4EBP1 phosphorylation (Inoki *et al.*, 2002). TSC2 phosphorylation activates its GAP (GTPase activating protein) activity which in turn converts Rheb into an inactive state (GDP-bound). This removes mitogenic stimulation. Rheb drives ribosome biosynthesis and hence global protein synthesis and is a key control point in both protein synthesis and cell proliferation rates (Dunlop and Tee, 2013). This is through Rheb binding to activating mTORC1 directly, associating with mLST8 and raptor, which is strongly inhibited by withdrawal of extracellular amino acids (Hara *et al.*, 2002; Long *et al.*, 2005)(Yadav *et al.*, 2013). Interestingly, this part of the TOR signalling pathway is conserved in *S.pombe* but not in *S.cerevisiae* although it has a Rheb protein it is not known to be involved in the TOR signalling pathway, therefore *S.Pombe* is used as a model organism to investigate this part of the TOR pathway (Aspuria *et al.*, 2007).

Peterson et al. (2009) discovered the protein DEPTOR (DEP domain-containing mTOR-interacting protein), a homologue of which has not yet been identified in yeast, that negatively regulates mTORC1. In 2007, Akt substrate 40 (PRAS40) was also discovered to inhibit mTORC1 kinase activity which depends on PRAS40 associating with raptor. When the expression of PRAS40 was reduced by short hairpin RNA it was found to result in an enhancement of 4E-BP1 binding to raptor. PRAS40 competes with 4E-BP1 binding to raptor, another mTORC1 component not found in yeast (Wang *et al.*, 2007).

6.1.1 TOR and amino acid sensing

TORC1 plays a major role in sensing amino acid availability in the environment, particularly leucine, as first discovered by Hall and colleagues (1996) and the absence of nutrients causes cells to enter stationary phase of growth (Barbet *et al.*, 1996). Just as in yeast, amino acid signalling and sensing is achieved in mammalian cells through a complex which interacts with the TORC1 complex which contains Rag proteins (Schurmann *et al.*, 1995; Sancak *et al.*, 2010). The Rag proteins are known as RagA and RagB (with 97.8% amino acid sequence identity) and are highly conserved (both are orthologues of Gtr1 in yeast). RagC and RagD (with 81.1% amino acid sequence identity) are also

similar (and have orthologue Gtr2 in yeast) and can pair with either partners forming a heterodimer (Schurmann *et al.*, 1995; Sekiguchi *et al.*, 2001; Kim *et al.*, 2008). RagC and RagD are related but it was found that as for Gtr1 in yeast, only RagA/B (GTP bound) is needed to maintain mTORC1 activity in an amino acid deprived environment and that amino acids regulate its nucleotide-bound state. RagA/B (GTP bound) may be the primary determinant for TORC1 signalling, however RagC and RagD are also essential for mTORC1 activation due to the instability of these dimers (Kim, Buel, & Blenis, 2013). Rag proteins may be unstable when not in the heterodimer complex as depletion of RagA and RagB causes a loss of RagC and RagD and vice versa (Sancak *et al.*, 2008). Unlike in yeast where Gtr1 can form a homodimer, this is not the case for RagA which is likely to be due to structural differences between the proteins (Sekiguchi *et al.*, 2001; Sancak *et al.*, 2008). Further, in contrast to mammalian cells where GTP-locked RagB uncouples mTORC1 from leucine signalling, GTP-locked Gtr1 only has a partial effect in yeast suggesting there may be other signalling routes involved (Binda *et al.*, 2009).

It has also been reported that RagC co-purifies with raptor, and that Rag GTPases binding to raptor is necessary to mediate amino acid signalling to mTORC1 causing the relocalisation to a membrane bound compartment containing Rheb (Sancak *et al.*, 2010). In starved HEK cells it was found that mTOR was in tiny punta throughout the cell, however upon addition of amino acids mTOR localises to the perinuclear region and large vesicular structures of the cell (Sancak *et al.*, 2008). It has been suggested that mTOR may interact with Rheb within the nucleus where several upstream regulators have been reported to be expressed (as well as the cytoplasm) regulating translation (Yadav *et al.*, 2013).

6.1.1.1 Regulation of Rag proteins

It is thought that the nuclear bound state of Gtr1 in yeast or RagA/B in mammalian cells is regulated by amino acid signalling, although the mechanism by which this is achieved is unclear (Kim *et al.*, 2013). In yeast Vam6 acts as a GEF controlling TORC1 activity by acting as a nucleotide exchange factor for Gtr1 and this is believed to be a homologue of Vps39. Δvam6 mutants show fragmented vacuolar morphology with defective protein processing suggesting this protein is required for the assembly of mature large vesicles(Nakamura *et al.*, 1997; Binda *et al.*, 2009). However, more recently Bar-Peled *et al.* (2012) demonstrated that Vps39 failed to interact with RagA, nor did it stimulate GDP or GTP dissociation from RagB. It was suggested this could be due to amino acid sensing mechanisms having diverged between yeast and higher eukaryotes.

In yeast Gtr1 and Gtr2 form part of the EGO complex for nutrient sensing involving Ego1 and Ego3 which tethers the Rag proteins to the vacuole. Subsequently it was discovered that these components

in TOR signalling were structurally conserved where Ego1 was similar to MP1 (mitogen-activated protein kinase scaffold protein1) and p14 (Kogan *et al.*, 2010). This complex was termed Ragulator by Sabatini and colleagues who later discovered it to be a pentameric complex with C7orf59 and XBXIP (hepatitis B X interacting protein), MP1, p14 and p18 which together controlled Rag lysosomal localisation in response to amino acids (Bar-Peled *et al.*, 2012). Although the Ragulator complex may be structurally similar to the EGO complex, Ragulator is also responsible for guanine nuclear exchange in RagA/B when amino acids are rich in the environment, activating RagA/B GTPases (Zoncu *et al.*, 2011; Bar-Peled *et al.*, 2012).

6.1.2 Autophagy

Cell growth and autophagy are important in the response to cellular nutrition status. During starvation, autophagy is important for recovering carbon source material to maintain cellular processes through a lysosome-mediated process in bulk degradation, breaking down old proteins, organelles and other components of the cytoplasm. This process is achieved through autophagosomes which engulf these components using special double membrane vesicles supplying the cell with an internal source of nutrients (Levine and Klionsky, 2004). The coordination of this process is through multiple ATG proteins first identified in yeast (summarized by Klionsky et al., 2013; Huang & Klionsky, 2002) as described in Chapter 5. In yeast Atg1 (ULK1 in mammals) plays a central role in autophagy, which is inhibited by TOR. When Atg1 is overexpressed, it induces high levels of autophagy, but Atg1 can also cause a negative feedback on the activity of TOR itself as cells with an autophagy defect had a growth advantage over wild type cells resulting in further activation of Atg1 promoting autophagy and reduced cell growth. However, S6K is also required for autophagy but is inactive when TOR signalling is lost (Scott *et al.*, 2007). Thus, processes are balanced with proper control through positive and negative feedback loops. Atg1 forms a complex with Atg13 (which is phosphorylated by TORC1) and during starvation Atg13 is partially dephosphorylated (Klionsky *et al.*, 2010).

Yan et al. (1999) identified and characterised ULK2 in mice, which has a 52% amino acid identity to ULK1, and suggested this is part of the TOR signalling pathway in mammals. It has been suggested that ULK1 and ULK2 may have originated by gene duplication during evolution. The role of ULK2 is less clearly defined at present, but ULK1 silencing is sufficient to inhibit autophagy (Chan *et al.*, 2007). A screen for ULK binding proteins discovered focal adhesion kinase family interacting protein of 200 kDA (FIP200) interacts with ULK1, this protein having a role in regulating cell size, proliferation and migration (Hara *et al.*, 2008). During starvation where TOR is no longer phosphorylating Atg13, Atg13

ULK1/2 and FIP200 complex recruits proteins to the phagophore including Beclin 1 which starts the induction of autophagy. Beclin 1 is important as it interacts with several enhancing or inhibitory factors which moderate its binding to Vps34 which is needed for vesicle nucleation/autophagosome formation (Kroemer *et al.,* 2010).

Table 6.1 List of TOR related proteins in budding yeast and mammalian cells discussed in this chapter. Not all pathways in TOR are as well conserved. * indicates yeast or mammalian protein with similar function but not involved in the TOR pathway.

Budding Yeast	Mammalian	Function
TORC1	mTORC1	TOR complex 1 involved in protein synthesis and ribosome biogenesis
TORC2	mTORC2	regulate the special aspects of growth including actin polarisation
TOR1,2	mTOR1,2	TORC1 component
Lst8	mLST8	Binds to TOR kinase domain required for TORC1/2 activity
KOG1	Raptor	TORC1 component, possibly couples TOR to substrates
Tco89	-	Involved in glycerol uptake under osmotic stress. Component of TORC1
-	Deptor	mTORC1 component, negatively regulates mTORC1
-	PRAS40	mTORC1 component, possibly associating with raptor
Atg1	ULK1,ULK2	Induce autophagy
Vam6	Vps39*	Assembly of large vesicles. In yeast, acts as GEF for Gtr proteins
Gtr1	RagA,B	GTP-binding protein involved in amino acid sensing
Gtr2	RagC,D	GTP-binding protein involved in amino acid sensing
Sch9	S6K	Major TOR protein kinase directly regulates protein synthesis
-	4E-BP1	Controls eIF4E in translation initiation
-	TSC1,2	Signalled via energy and growth factors, directly acting controlling Rheb downstream
RHB1*	Rheb	Rheb activates TORC1. Both Rheb and RHB1 regulate arginine uptake
Snf1	AMPK	glucose sensory protein kinase
Ego1,Ego3	Regulator	Tethers Rag proteins to lysosome/vacuole. Ragulator may also act as GEF for the Rag proteins in mammals

6.1.3 CHO cells as a model mammalian cell system and for the production of biotherapeutic recombinant proteins

A variety of organisms including yeast and *E.coli*, and cultured cell systems, are used to produce recombinant proteins for clinical applications, however proper folding, assembly and human-like post-translational modifications are important for correct protein function in humans and this is superior in mammalian cells (particularly human-like glycosylation) (Wurm, 2004) as outlined in the introduction chapter of this thesis. Chinese hamster ovary (CHO) cells in particular are used for producing recombinant therapeutic proteins as they can be genetically manipulated producing complex therapeutics with manufacturing adaptability, delivering up to several grams per litre of products with a highly optimised production process (Xu *et al.*, 2011). However, with the continued development of new protein based biopharmaceutical target drugs, there is a continual need to further improve titre and particularly quality (correct folding and post-translational modifications) of biotherapeutic proteins from mammalian cells (Walsh, 2010a). TOR signalling in particular has been shown to improve antibody titre by four-fold by ectopic expression of the global metabolic sensor mTOR, improving cell size, proliferation, viability, and robustness of the cell (Dreesen and Fussenegger, 2011). As TOR is conserved in yeast and higher eukaryotes such as CHO cells, here we investigated how disrupting TOR signalling via the specific TOR targets investigated in the yeast system described in Chapter 5 of this thesis translated into CHO cell phenotypes was investigated, particularly the effects on recombinant protein production using the Guassia luciferase reporter protein as a model system, as utilised in the earlier yeast studies described in previous Chapters of this thesis.

Results

6.2 Investigation and manipulation of TOR signalling in CHO cells and its effect on recombinant protein production

From the results described in chapter 5, the yeast genes from the EGO complex Gtr2, and Vam6 which acts as a GEF for the GTPase were selected for further study in CHO cells, this complex being responsible for amino acid sensing in TOR. Although the heterodimer to Gtr2, Gtr1, is also part of the complex, due to time constraint, this gene was not investigated. Deletion of *GTR2* caused a loss of rP production, although the deletion of VAM6 had no effect we chose to test this in the mammalian system due to the lack of knowledge of the protein in this role. The mammalian orthologues for these

genes Vps39 (Vam6), and RagC and RagD (Gtr2) were investigated in the CHO system (Schurmann *et al.*, 1995; Sancak *et al.*, 2010).

From the yeast screen data, Atg1 caused a loss in rP production and has two mammalian homologues; ULK1 and ULK2, however it was previously reported that the silencing of ULK1 was sufficient to inhibit autophagy, therefore it was decided to knockdown the gene for ULK1 in CHO cells (Yan *et al.*, 1999; Chan *et al.*, 2007).

6.2.1 Effects of the knockdown of selected TOR targets in CHO in an initial screen

To test if similar effects on GLuc expression from CHO cells were observed as to that observed in yeast in chapter 5 when manipulating TOR gene expression, an adherent CHOK1 cell line which expressed *Gaussia* luciferase (GLuc) previously generated at The University of Kent was utilised. GLuc is a naturally secreted protein from the organism *Gaussia principes* which is used as a molecular tool to study gene expression (Ruecker *et al.*, 2008). Here, how alterations to TOR signalling effect GLuc secretion in the model CHO system was investigated. The GeneClip U1 Hairpin Cloning System from Promega was used to develop tools for the specific knockdown of target genes, thus achieve silencing of these TOR genes, as described in the methods chapter. This approach uses a foldback stem-loop structure which suppresses the expression of the target TOR gene sequence. This structure stimulates specific degradation of the target mRNA (Zamore, 2001; Wu, 2009).

Oligonucleotide design was undertaken to produce two hairpin oligonucleotides which anneal to form a double-stranded DNA fragment containing a target sequence specific to each selected TOR gene. After the annealing reaction, the double-stranded DNA fragment containing a *Pst1* site was inserted into the pGeneClip vector by the overhang sequence which was complementary to the vector overhang. These vectors containing the hairpin were transformed into competent *E.coli* cells, with the DNA extracted and sequenced to identify correct clones. CHOK1-GLuc cells were then transfected with the vectors for transient expression/knockdown and GLuc expression measured after 24 and 48 hours of knockdown by western blotting. This approach allowed for the determination of the 'best' conditions for knockdown of mRNA of the selected TOR genes as optimal conditions can vary. The amounts of mRNA knockdown were verified using qRT-PCR where mRNA levels were compared in the control to TOR knockdowns. For optimisation initial data is reported in Figure 6.2. Note that no housekeeping genes were used for normalisation in this experiment.

A)
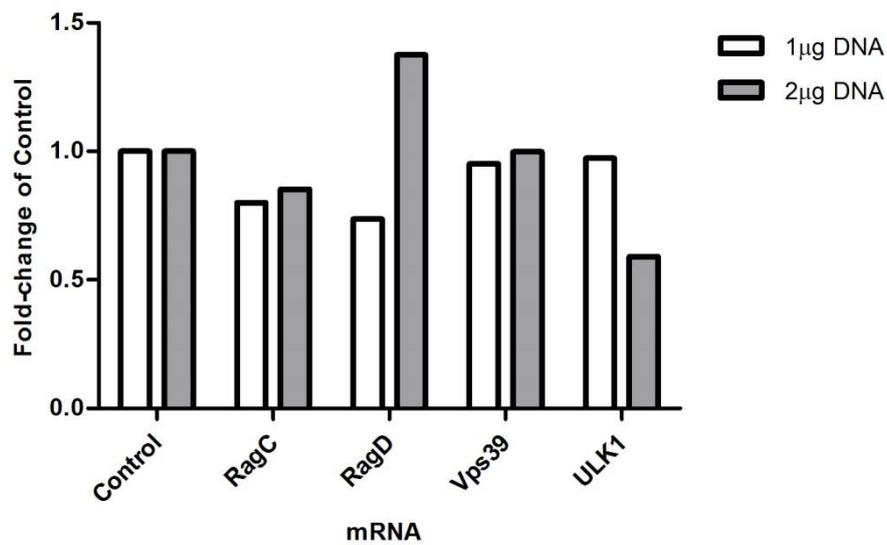
B)
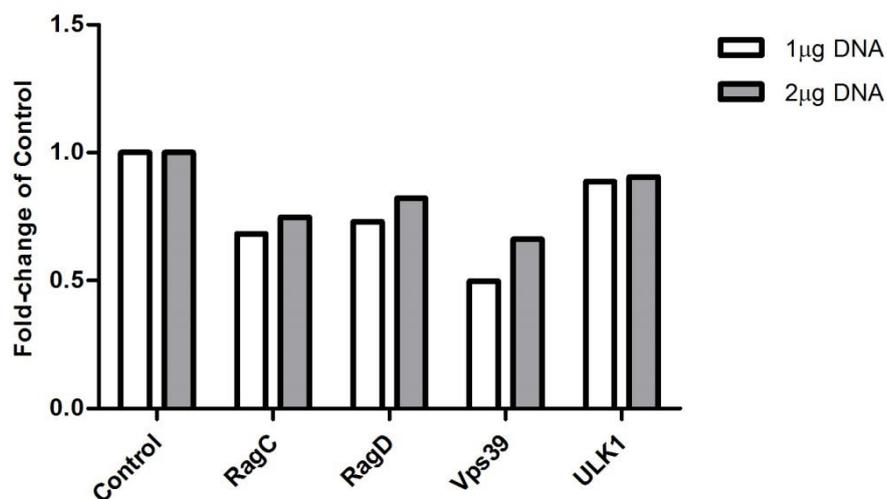
 Figure 6.2 mRNA levels of TOR knockdowns relative to the control. In 6 well plates 5×10^5 viable cells were seeded the day before transfection with the vector (n=1). Control contains scrambled sequence in the vector transfected. Half the cells grown were re-suspended in 350 µl RLT and RNA extracted. This was then DNAse treated before used for qRT-PCR. A) mRNA levels after knock down relative to the control in cell lines after 24 hours with 1 µg and 2 µg DNA. B) mRNA levels after knock down relative to the control in cell lines after 48 hours with 1 µg and 2 µg DNA. (n=2, technical replicates). Note cells taken for each time point were from different wells. qRT-PCR was measured using Quantifast SYBR Green PCR kit and run using MiniOpticon System (BioRad). Note that no housekeeping genes were used for normalisation in this experiment.

Levels of mRNA detected in knockdown transfected cells were compared to that in the control with transient expression of the hairpin containing vector. After 24 hours the knockdown in some straing were 20% or less; however there was an increase in the knockdown observed after 48 hours except for the knockdown of ULK1, which was still low. The level of knockdown at the mRNA level was not considered high (>70%) for any of the genes with the knockdown of Vps39 mRNA showing the greatest knockdown of about 40% 48 h after transfection of the shRNA plasmid. Surprisingly, after 24 hours with 2 µg DNA added, an increase in RagD mRNA levels was observed, with a knockdown only observed after 48 hours. The general trend observed was that after 48 hours there was an improved knockdown of all mRNA from the shRNA with 1 µg DNA and this may be due to the time required to synthesise the shRNA and then subsequently for the mRNA to be targeted and degraded. Regardless, although the level of knockdown was not optimal (considered to be >70%), due to time limitations further work was undertaken to determine if this modest knockdown at the mRNA level resulted in any changes to recombinant protein expression. As antibodies to the proteins coded by these mRNAs were not available it was not possible to determine if the modest mRNA knockdown subsequently resulted in reduced protein levels at the time points investigated.

Protein cell lysates were extracted and prepared from the same wells as those where mRNA levels were determined after knockdown to measure GLuc expression at the same time points. GLuc and actin antibodies were then used to assess recombinant protein production in the intracellular samples with selected TOR genes knocked down by western blot analysis and for GLuc in the supernatant (secreted recombinant protein) (figure 6.3).

A)

B)
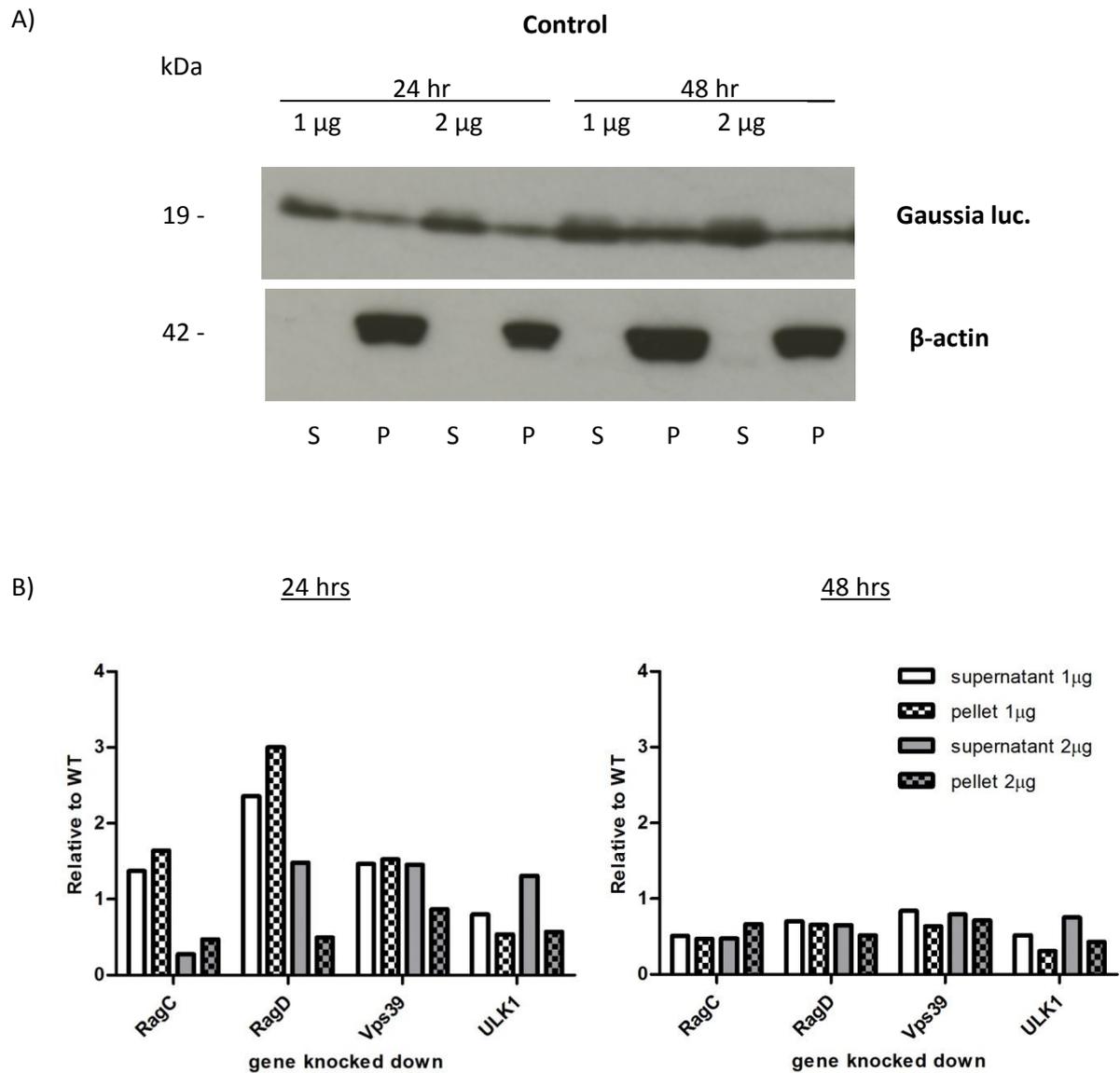
Figure 6.3 Initial screen of GLuc and actin expression in CHO cells and of GLuc in the cell culture supernatant with selected TOR knockdowns. In 6 well plates $5x10^5$ viable cells were seeded the day before transfection with the vector. Control samples contained scrambled sequence in the vector transfected. 1 ml supernatant was removed with 200 µl lysis buffer added. Half the cells grown in each plate were collected by centrifugation and treated with 200 µl of lysis buffer. (n=1) The Bradford assay was performed to measure protein levels. A) GLuc expression time course at 24 hours and 48 hours using 1 µg and 2 µg control DNA in the supernatant (s) and pellet (P). B) Quantification using Image J of GLuc expression in supernatant and pellet after 24 h and 48 h using 1 µg and 2 µg DNA for TOR knockdowns. (n=1). Quantification was undertaken using the software Image J.

GLuc expression detected in the supernatant and pellet following targeted knockdown is reported in figure 6.3a where expression was determined 24 and 48 hours after transfection with the scrambled sequence as a control. This data illustrated that there was more GLuc expressed from CHO cells

compared to yeast (chapter 5) with more found in the supernatant at both 24 and 48 hours. As expected, more GLuc was detected after 48 hours than 24 hours of culture and the control DNA amount transfected made little difference to secretion.

The amount of GLuc in the various knockdowns 24 hours after transfection with 1 µg DNA is reported in figure 6.3B. An increase in GLuc expression was observed in RagC, RagD and Vps39 knockdowns at 24 h, yet with 2 µg generally a decrease in expression was observed. In the knockdown of both Rag proteins higher GLuc expression with 1 µg DNA and greater knockdown was observed suggesting that knockdown of these mRNA may result in an increase in GLuc protein production. Knockdown of RagD with 1 µg in particular resulted in an approximate 3-fold increase in GLuc expression in the pellet and slightly less in the supernatant. Although the level of mRNA knockdown of RagD and in RagC was comparable at 24 hours, RagD knockdowns had more apparent effects on the GLuc expression levels. This could be due to a smaller level of mRNA knockdown producing a bigger change in protein expression in RagD although this was not investigated here as antibodies to Rag D were unfortunately unavailable. Transfection of the hairpin for RagD could ultimately result in a different effect in the cells compared to the hairpin for ULK1. ULK1, showed a higher percentage knockdown of mRNA with 2 µg DNA, yet showed only marginally higher levels of GLuc secretion in the supernatant. This was the only mRNA targeted which had more knockdown with 2 µg of DNA.

Vps39 knockdown showed little change to GLuc expression after 24 hours and there was also little change to mRNA levels which suggests the knockdown was not efficient in this transfection or that the sequence designed for the knockdown did not result in the desired knockdown. When comparing GLuc expression with the varied amounts of DNA little change to mRNA levels was observed yet a reduction in GLuc expression with 2 µg DNA after 24 hours was detected suggesting this amount of DNA was negatively influencing the cell's ability to produce recombinant protein.

We discovered 48 hours after transfection, there was a general decrease in GLuc expression across all knockdowns with both amounts of DNA investigated in the pellet and supernatant suggesting the cells responded differently after 24 hours and 48 hours to the transfection (figure 6.3C). As there was a drastic change to GLuc expression in RagC after transfection with both 1 µg and 2 µg of DNA and in RagD with 1 µg DNA, this amount was used in future experiments and GLuc expression monitored after 48 hours transfection/knockdown.

From these initial studies, knockdown from transfecting the shRNA was achieved but only at modest amounts of 30-40% mRNA knockdown and there was a higher degree of knockdown after 48 hours

with 1 µg DNA (see figure 6.2). Knockdown of all the targets except ULK1 mRNA caused an increase in GLuc expression after 24 hours then a decrease in all knockdowns after 48 hours (figure 6.3).

6.2.2 Effects on recombinant protein production of Rag protein knockdowns

The data presented in figure 4.2 and 4.3 illustrate knockdown, albeit very modestly, using 1 µg of the vector containing the hairpin after 48 hours achieves the highest knockdown. As knockdown of these selected TOR genes reduced rp production at 48 h, we wanted to confirm this by repeating the transfection of the vector silencing RagC and RagD as one of the main components of nutrient sensing. This test would also confirm if perturbing nutrient sensing in yeast via the GTPases in the EGO complex gives the same effect in CHO cells when analysed by monitoring GLuc expression. In addition, a double knockdown of RagC and RagD was also performed, as only the knockdown/silencing of both would compare to deletion of Gtr2 in yeast. Figure 6.4 below reports the mRNA levels detected after 48 hours post transfection with the shRNA using 1 µg of DNA. As described earlier, qRT-PCR was undertaken to determine target gene mRNA levels, and hence knockdown, from cells grown in 6 well plates.
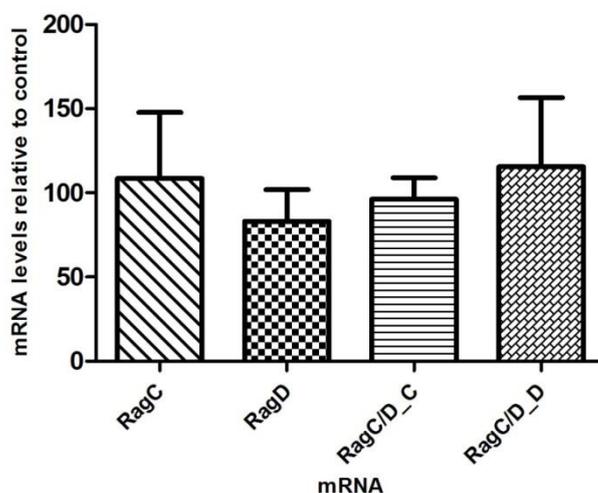
Figure 6.4 mRNA levels of Rag knockdowns after 48 hours. Percentage of mRNA levels in cell lines relative to the wild type after 48 hours knockdown with 1 µg DNA. In 6 well plates $5x10^5$ viable cells were seeded the day before transfection with the vector. Control contains scrambled sequence in the vector transfected. Half the cells grown were resuspended in 350 µl RLT and RNA extracted. This was then DNAse treated before used for qRT-PCR. qRT-PCR was measured using Quantifast SYBR Green PCR kit and run using MiniOpticon System (BioRad). Statistical analysis was performed using one-way ANOVA test (*$P<0.05$, **$P<0.01$, ***$P<0.001$). (n=4) Errors bars represent standard deviation.

mRNA levels were measured after 48 hours of transient knockdown of selected TOR mRNA. However it was revealed that little/no changes to mRNA levels suggesting there was no knockdown of the target genes in this experiment at 48 h post-transfection (figure 6.4). Despite this, GLuc expression was still measured to determine if the transfection itself of the different knockdown plasmids had influenced rP GLuc expression. GLuc expression is reported in figure 6.5.

A)

B)
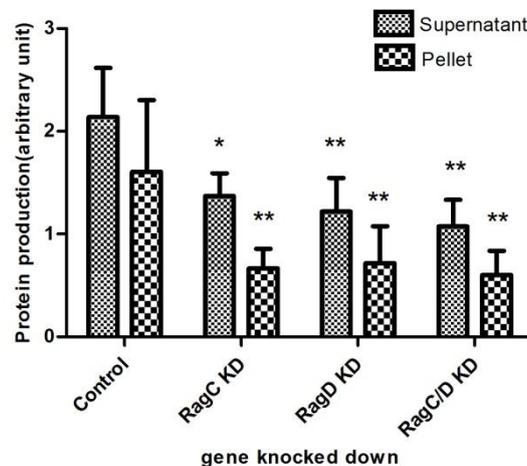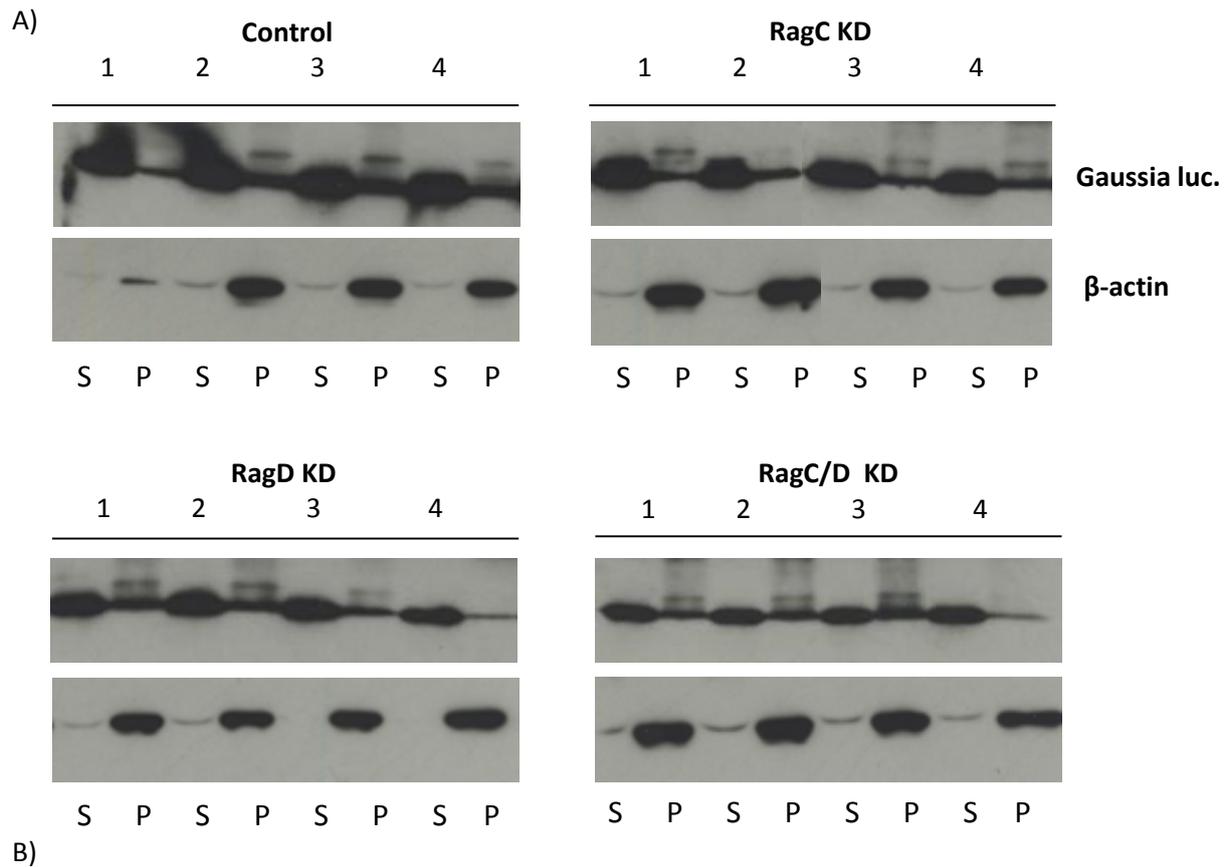
Figure 6.5 GLuc expression in cell lines 48 hours after knockdown. Actin expression was also detected for normalising data. In 6 well plates 5x10^5 were seeded the day before transfection with the vector. Control contains scrambled sequence in the vector transfected (KD-knockdown). 1ml supernatant was removed with 200µl lysis buffer added. Half the cells grown in each plate were spun down and treated with 200µl of lysis buffer. (n=1) The Bradford assay was performed to measure protein levels. a) Gluc expression in control and Rag knockdowns after 48 hours using 1 µg DNA in supernatant(S) and pellet (P). b) Quantification of GLuc expression in knockdowns after 48 hours using 1 ug DNA (n=4). Quantification was done using the software Image J. Statistical analysis was performed using the ANOVA test (*P<0.05, **P<0.01) which compares GLuc in the supernatant of each strain to the control as well as GLuc detected in the pellet to the control. Errors bars represent standard deviation.

It was revealed that GLuc is secreted in higher amounts in the control than in the knockdown transfected samples (figure 6.5A), as observed before in figure 6.3A. Quantification of these blots in figure 6.5b showed that transfection with the knockdown plasmids caused a statistical decrease in secretion in RagC ($P<0.05$), RagD ($P<0.01$) and knockdown of both combined ($P<0.01$). This was a surprising result as there was little/no knockdown detected at the mRNA level as reported in figure 6.4. Either there was a subtle but non-detectable knockdown, knockdown had occurred earlier and then recovered or the transfections were influencing the cells in some other, unknown manner causing a reduction in GLuc protein synthesis within the cell. We also observed a reduction in GLuc trapped in the pellet when comparing all knockdowns to the control with the transfection of the plasmid for knockdown of RagC caused a significant loss of expression ($P<0.01$), in addition a loss with the knockdown of RagD ($P<0.01$). When comparing transfection of the plasmids for the knockdown of RagC and RagD, there was little difference between these when measuring GLuc expression. With the double knockdown transfection we saw a similar reduction to GLuc expression suggesting that transfection of both plasmids caused no further perturbation to rP production.

In conclusion, the data here is inconclusive as to whether Rag proteins are important in rp production in CHO cells as determined from GLuc expression as insufficient knockdown of the target mRNAs was achieved. The fact that GLuc expression decreased upon transfection does agree with the data earlier in this chapter but the lack of knockdown precludes any conclusions being drawn with respect to the effect of reducing amino acid sensing and rP production as reported in the yeast with the deletion of Gtr2. Of course, amino acid sensing may not be relevant at 48 h after transfection when sufficient nutrients are still available in the media and longer time points may have been more interesting if sufficient knockdown could have been achieved.

6.3 Discussion

In this chapter we investigated TOR signalling in the CHO expression system in relation to rp production using CHOK1 cell line expressing the secreted protein GLuc. We previously identified TOR proteins in yeast from a screen which caused a loss to GLuc expression when these genes were deleted; as CHO cells are predominantly used in the biopharmaceutical industry we wanted to test if we detected the same effects to GLuc protein synthesis when the mammalian counterpart is silenced.

An attempt at silencing of these selected mTOR mRNA was performed using shRNA which targets the mRNA for degradation. In the initial test (figure 6.2) we detected a higher percentage knockdown in general after 48 hours with 1 µg DNA as this gives more time for the vector containing the shRNA to be expressed and target the specific gene by the shRNA for degradation by the enzyme Dicer (Zamore, 2001; Wu, 2009). A vector containing a scrambled sequence was used as a control and its GLuc expression measured where we observed more GLuc secreted versus that in the cytoplasm (figure 6.3A). Compared to what was detected in yeast, CHO are much better secretors of GLuc, however the activity of the protein secreted was not tested in both systems.

With GLuc expression quantified, we observed a loss in GLuc expression detected in the pellet and supernatant across all knockdowns (figure 6.3C). Unfortunately it was not possible to confirm if the modest knockdowns at the mRNA level had resulted in a knockdown, and to what extent, at the protein level due to a lack of available antibodies.

6.3.1 Loss of autophagy in CHO cells

Autophagy is inhibited downstream of TORC1 through ULK1/2 which forms a complex with ULK13 and FIP200 (Hara *et al.*, 2008). ULK1/Atg1 is a major coordinator of autophagy which is essential for recycling old or damaged components in the cell increasing intracellular nutrients during starvation or other forms of environmental stress and so is necessary for efficient protein synthesis in both yeast and CHO cells (Levine and Klionsky, 2004; Ganley *et al.*, 2009). To test the role of ULK1 in GLuc production, its gene was knocked down using shRNA. A low percent knockdown of ULK1 mRNA using shRNA was achieved (figure 6.2), yet we observed a similar loss of GLuc expression after 48 hours transfection (figure 6.3C). This suggested that the sequence targeting ULK1 worked in the shRNA, and a loss of ULK1 (although not fully knocked out) resulted in an impact upon rp production.

Of all the autophagy genes, some regulate development in other pathways in higher eukaryotes including Beclin 1 (Atg6 in yeast) which interacts with anti-apoptotic members of the Bcl-2 family (Levine and Klionsky, 2004; Lee *et al.*, 2013). CHO cell phenotypes grown in industry in large batches to produce rP can be improved with cellular engineering, for example ectopic expression of mTOR which increased antibody production (Dreesen and Fussenegger, 2011). Kroemer *et al.*, (2010), discuss how autophagy integrates stress responses which would accumulate during cell growth such as ROS, DNA damage, damaged organelles and protein aggregates.

As CHO cells are engineered to produce rP in high volumes, there is added stress such as ER stress leading to misfolded protein and mitochondrial dysfunction which can result in cell death by apoptosis (Bravo *et al.*, 2011). Autophagy can however be harnessed as an anti-cell death target in conjunction with controlling apoptosis during stressful culture conditions. For more efficient protection of cells, apoptosis and autophagy are both being targeted in CHO cells. For example, Bcl-2, an anti-apoptotic protein and Beclin-1, a key regulator of autophagy were co-overexpressed causing a synergistic effect by enhancing anti-cell death during growth (Lee *et al.*, 2013). One of ULK1's role is in phosphorylating Beclin-1 to initiate autophagosome formation, which supports the phenotypic response found here from silencing ULK1(Russell *et al.*, 2013).

6.3.2 Inhibition of amino acid sensing

RagC/D (Gtr2) form a heterodimer with RagA/B (Gtr1) which form part of the EGO complex which needs the GEF Vps39 (vam6), important for activating TORC1 and controls growth and protein synthesis (Kim *et al.*, 2008). rP production places additional energy demands upon the cell and therefore nutrition sensing is important in optimising recombinant protein synthesis. It is known that glucose and energy sensing is undertaken via AMPK, yet amino acid sensing is poorly understood. Amino acids are essential for mTOR activation because even growth factors and other stimuli are not efficient in activating mTOR when amino acids are limited (Jewell and Guan, 2013).

The knockdown using shRNA confirmed a loss of GLuc expression (figure 6.3) in RagC and RagD (Gtr2 in yeast) and Vps39 (Vam6 in yeast), confirming their importance in rP production. We reported a loss of GLuc expression (figure 6.3) when only limited silencing of Vps39 was achieved (figure 6.2). Although Vps39 was previously believed to be the GEF for the Rag proteins, it was recently demonstrated that Vps39 does not interact with RagA suggesting that the effects of silencing Vps39 are not due to the loss of amino acid sensing but only due to its role in the assembly of vacuoles, a

function already known (Nakamura *et al.*, 1997; Binda *et al.*, 2009; Bar-Peled *et al.*, 2012). In yeast it was discovered that Vam6 has a function in the vacuolar assembly and deletion of this gene causes vacuole-related structures smaller than normal vacuoles(Nakamura *et al.*, 1997; Binda *et al.*, 2009). The loss of this function in CHO cells may have been what was causing loss of GLuc expression. Vps39/Vam6 is known to co-localise with Vps41/Vam2 (also involved in vacuolar assembly) and form part of the HOPS complex which is required for several fusion events at the late endosome and the vacuole including the fusion of autophagosomes and golgi vesicles (Bröcker *et al.*, 2010). Quality control is important in the secretory pathway where substrates targeted for degradation can be targeted for the endosomal system for lysosomal degradation, this is important for normal protein trafficking and stability in the secretory pathway (Arvan *et al.*, 2002; Brodsky and Skach, 2011).

The attempted silencing of RagC and RagD was repeated but with more replicates, and again a loss to GLuc expression was observed when they were knocked down separately or together (figure 6.5). It was not unsurprising that the knockdown of each caused similar alterations to GLuc production as they are functionally redundant (Schurmann *et al.*, 1995; Sancak *et al.*, 2010). Unfortunately the knockdown of these mRNA proved difficult to achieve, the reasons for this are not clear at this stage (figure 6.4). When transfecting with shRNA for both RagC and RagD a further decrease to GLuc expression was observed, yet not all function is lost. Although there was little/no change to mRNA levels observed, the system was perturbed causing a loss in GLuc expression detected in both the supernatant and the pellet. The reason for this is unclear but may be the result of either off target effects, recovery of mRNA levels at 48h after knockdown or the DNA itself may have been toxic to the cell and generally slowed protein synthesis (although this was not seen in the initial knockdown, figure 6.3). A future experiment would be to check the protein levels of RagC/D, Vps39 and Ulk1 as we did not have the antibodies available. It would be interesting to see if knockdown of RagA/B shows the same effect on GLuc production, and even further the combination knockdown of RagB/C. It was found that RagB-GTP/RagC-GDP was the most effective in signalling to mTORC1 (Sancak *et al.*, 2010).

Interestingly, GLuc expression investigated in RagC, RagD and Vps39 knockdowns had shown an increase in GLuc expression from RagD knockdown in particular (figure 6.3b). There are a number of possible reasons to explain this increase after 24 hours of transfection before a reduction in GLuc at 48 hours. The transfection process itself may have caused an effect resulting in either stress or some form of cellular response causing a transient increase in expression before the knockdown at 48 hours. There is literature discussing how lipofectamine used for siRNA transfections can cause an increase of autophagasomes (Mo *et al.*, 2012). Any stresses can cause cellular changes, however knockdown of these three mRNAs in this particular part of the TOR pathway may be more sensitive. Another reason

could simply be due to the initial response of the knockdown resulting in the GLuc increase but after a longer time period TOR signalling is shutdown reducing rP production. Further experiments would need to be undertaken to confirm whether any of these mechanisms were responsible for the observed GLuc expression profiles.

6.3.3. Conclusion

In summary, the control of TOR signalling through various upstream and downstream processes is paramount for cellular homeostasis during growth. The nutrient status of the cell is carefully monitored by the Rag complex in communication with mTORC1 via raptor which is necessary for efficient rP production. However, this process along with other control mechanisms in the cell works together to correctly sense and react to the appropriate cues from intracellular and extracellular senses. This chapter investigated how both amino acid sensing as well as autophagy, which promote and inhibit TOR signalling (via feedback mechanisms) respectively, reduces GLuc expression when silenced in CHO cells (Dunlop and Tee, 2013). Unfortunately the hairpin approach to knockdown target gene expression was largely unsuccessful. The reason for this are unclear as this system has been used successfully to knockdown other target genes within our research group. Further investigation is needed to determine if manipulating these selected components of mTOR signalling by overexpression could potentially enhance rP production which would be beneficial to industry. Improved knockdown and protein levels detected for further confirmation of the effects of the manipulation of these genes needs to be undertaken to allow a full assessment of the effects of reducing these key mTOR players on recombinant protein expression in CHO cells.

Chapter 7

Final Discussion

7.1 Summary

Since the advent of recombinant DNA technology and the approval of the first rP for use in humans by the FDA around 30 years ago, heterologous protein production has been possible in a variety of systems including plants and cultured yeast, bacteria, insect and animal cells. Advances in recombinant DNA technology alongside an ever improving knowledge of the mechanisms involved in controlling protein synthesis, quality control, responses to environmental conditions, coupled with genetic manipulation of host expression systems have been instrumental in boosting rP productivity (Ferrer-Miralles *et al.*, 2009). Through the genetic engineering of various organisms and cell systems, it is possible to improve production rates by harnessing improved cellular functions. Examples include altering protein processing through the secretory pathway (such as the overexpression of the PDI folding protein), metabolic engineering for improving growth and viability, and use of anti-apoptotic strategies to prevent cell death in the bioreactor (Davis *et al.*, 2000; Fussenegger *et al.*, 2000; Palomares *et al.*, 2004; Dinnis and James, 2005).

Due to the relative ease of genetic manipulation and fast growth, yeast are common manufacturing systems in rP expression of biotherapeutics (16.5% of approved products are expressed in yeast), including the use of *S.cerevisiae* and *P.pastoris* (Walsh, 2014). In this study we harnessed *S.cerevisiae,* one of the most well-studied lower eukaryotic microbial systems, as a model for the expression of recombinant reporter molecules. In addition, we used this to probe cell signalling pathways and the influence on rP production. Yeast are able to adapt to stresses such as low oxygen levels, high osmolarity and temperature by adapting to new conditions through transient responses of gene expression changes (Mattanovich *et al.*, 2004; Porro *et al.*, 2005; Rodríguez-Peña *et al.*, 2010; Çelik and Çalık, 2012).

Specifically, *S.cerevisiae* was utilised in this project to investigate how modifications to various nutrient sensors in the cell may affect rP production, as cell growth is controlled by the cellular responses to nutrient availability and stress through various signalling pathways. However, due to the complex nature of the mechanisms involved through cell signalling, cross talk between pathways does occur. For example, there is considerable cross-talk between Target of Rapaymcin (TOR) and the PKA pathway in controlling autophagy when nutrients are scarce (Stephan *et al.*, 2014). As the TOR pathway is well conserved among eukaryotes, the aim here was to investigate the yeast system and then establish how these findings could be related to the mammalian expression system, Chinese hamster ovary (CHO) cells. This is a robust system most commonly used in expressing rP due to their ease of manipulation, robust performance in the bioreactor, ability to be cloned to deliver cell lines

with different phenotypes, regulatory approval and ability to fold and assemble complex proteins with human-like post-translational modifications, particularly glycosylation (Fan *et al.*, 2012; Walsh, 2014).

In this thesis, a reporter construct for expressing the heterologous protein *Gaussia* luciferase (GLuc) from the organisms *Gaussia princeps* was used as a model recombinant expression molecule and the signal sequence from the yeast pheromone gene MFα was used for the secretion of the model recombinant protein (Lin-Cereghino *et al.*, 2013). The advantage of using this construct in yeast is that it allows the user to rapidly screen a variety of mutants with altered nutrient and stress signalling to determine their influence on rP production. However, before undertaking the screen, it was necessary to identify and optimise the screening process using the GLuc construct. The results from this process are reported in Chapter 3, and a discussion of the results is presented below.

7.2 Optimisation of the yeast model system using the GLuc construct

The aim of the work described in Chapter 3 was to find a suitable screening method for identifying genes/proteins that may influence recombinant protein expression, as determined using a GLuc reporter construct developed specifically for yeast. The GLuc assay, involving measuring varying luminescence between strains depending on their ability to produce rP, was investigated; the pH of the media and GLuc expression (through a time course) were determined. Although luminescence was assumed to be stable under varied pH conditions, low or high pH may influence the yeast mutants, modifying/inactivating enzymes involved in processes along the secretory pathway (Figure 3.5) (Wiles *et al.*, 2005; Tannous *et al.*, 2005). There was significant variation in GLuc activity detected from day to day, despite following a defined methodology (Figure 3.7). This work suggested that the GLuc activity assay was not robust under the conditions investigated and could not be used for reliable comparisons across experiments and different days to determine effects of gene manipulation on expression (Figure 3.7). An option could have been to use an injector so the readings were taken immediately after the substrate is added, as the timing may have been introducing this error when signal is lost. However, an alternative method of assessing protein expression was investigated.

As an alternate method, western blotting using a GLuc antibody for determining expression levels was investigated, allowing determination of the recombinant GLuc protein levels in the pellet and the supernatant. This provided additional information as to how mutations may affect protein processing as well as secretion (Figure 3.8). The disadvantage of this approach is that the method determines protein amounts, not activity, and as such manipulation of a specific target gene/protein may

increase/decrease total protein expressed but influence the amount of active protein present in a different manner (e.g. more protein might be expressed but at the expense of folding and as such the change in the amount of active protein may not reflect the change in the amount of total protein). Taken together, this suggests that the system used has variable quality output, since measuring protein production in this way gives accurate results but variable activity measurements. One improvement to overcome this issue may be to investigate activity levels in yeast with a different genetic background which may more accurately produce a functional product. Mutations in the protein cofilin for example, causes alterations to the protein's role as a homeostatic regulator, therefore these genetically altered cells may be causing unstable activity of the protein.

In addition, an alternative rP system was also established to ensure that any changes observed were not specific to the GLuc construct. The signal sequence from the yeast mating pheromone is commonly used for secretion (Lin-Cereghino *et al.*, 2013). As an alternative screen, the Killer Toxin assay was utilised which is a viral model system also used previously to target foreign proteins for secretion (Schmitt and Breinig, 2002). We predicted that as the rP is driven by a different signal sequence, the secretion profile of the wild type and mutants investigated may be different under varied conditions. As this protein assay is inexpensive and rapid, the assay has proven to be a good tool for screening. Nonetheless, this assay relies on a low pH media for the toxin to function and therefore the observed results may be influenced as altered strains may adapt to the environment differently as pH stress will alter signalling activity differently with mutated components. As such, both CHO and yeast systems were selected to screen for gene/proteins whose manipulation changes rP production through alterations to stress sensing through genetic mutations. As TOR signalling is so well conserved between the two systems, we discovered similar results in chapter 5 (yeast cells) and chapter 6 (CHO cells) in investigating altered amino acid sensing and its importance in rP production (see section 7.4.1 for more detail).

7.3 The actin binding protein Cofilin plays a role as an environmental sensor revealing the importance of mitochondria in rP production and stress signalling in yeast.

7.3.1 Linking mitochondria and cofilin in rP production

Unpublished data from the Gourlay lab at the start of this project highlighted the importance of mitochondrial function for rP production. When comparing a high and low rP producer of albumin and transferrin, Gourlay *et al* found the higher producer had greater mitochondrial damage, as determined by respirometry studies (unpublished data). It is well established that mitochondria are important in sensing and regulating ER stress, controlling apoptosis through the unfolded protein response (UPR), as well as supplying the energy required during protein synthesis. These organelles communicate through mechanisms of calcium transfer via the ERMES (endoplasmic reticulum(ER)-mitochondrial encounter structure) junction, which is important in modulating mitochondrial bioenergetics for the cell to adapt during stress (such as that encountered during rP production) (Bravo *et al.*, 2012). Although this is only true in yeast, mammalian cells have a functional equivalent (described in chapter 1 section 1.3.2.5) (Kornmann and Walter, 2010).

Cofilin, the actin binding protein, is known to translocate to the mitochondria during oxidative stress and initiate the release of cytochrome C leading to apoptosis (Liu *et al.*, 1996; Chua *et al.*, 2003). It was recently reported that specific mutations outside of cofilin's actin binding region leads to increased mitochondrial function in yeast (Kotiadis *et al.*, 2012). We hypothesised that such cofilin mutations may be beneficial in terms of subsequent effects upon rP production yield. As ER stress causes mitochondrial dysfunction leading to cell death (Lim *et al.*, 2009), enhanced mitochondrial function may buffer against this. In CHO cells, the key signalling pathway TOR (which controls growth and protein synthesis through the TORC1 complex), is also involved in regulating mitochondrial biogenesis. This is achieved via PGC1a, a transcriptional activator of genes encoding proteins in mitochondrial function in mammalian cells. Although there is no known orthologue in yeast cells, this increase in mitochondrial biogenesis may be advantageous for rP production.

As enhanced mitochondrial biogenesis may be beneficial to rP production, we sought to investigate if recombinant GLuc expression could be effected by changes to cofilin that altered mitochondrial function. In order to address this, a library of cofilin mutant alleles were expressed to determine if modification of specific residues outside of the actin binding region with alanine mutations, removed/changed the function of the protein and therefore rP production (Lappalainen *et al.*, 1997).

These mutant alleles were split into three groups depending on effects upon mitochondrial biogenesis or actin organisation (Kotiadis *et al.*, 2012). Class I contained *cof1-4, cof1-6, cof1-7, cof1-11, cof1-12, cof1-15, cof1-18, cof1-19, cof1-21* expressing strains which all demonstrate an increase in mitochondrial respiration (Kotiadis *et al.*, 2012). Class II included *cof1-10* and *cof1-13* which possessed slow growth, decreased respiration and impaired actin regulatory function; compared to Class III (*cof1-5, cof1-8 and cof1-22*) with severely altered actin organisation as well as temperature sensitive growth (Lappalainen *et al.*, 1997).

When expressing GLuc in a selection of these strains with varied mitochondrial function (class I that have enhanced mitochondrial function), cells expressing the cofilin mutant allele *cof1-21* showed an increase in GLuc secretion compared to all other strains. This suggests that the mutated protein positively impacts upon rP production (Figure 4.3). We found that the loss of mtDNA in the wild type cells led to an almost complete loss of GLuc expression. However, GLuc expression was apparent in cells expressing cofilin mutant alleles. This data suggested that signalling between cofilin and the mitochondria is important in determining rP production. When position 103 containing an aspartic acid is mutated to alanine an advantageous response in rP production is observed, although the mechanism by which this results in this phenotype is unknown. As already discussed, mitochondria are important in regulating the metabolism of the cell especially during rP production, where the cell is forced to use more energy and nutrients. With the loss of mitochondrial function and metabolic insufficiency, the ER may become stressed leading to cell death (Bravo *et al.*, 2012). mtDNA is needed for glutamate biosynthesis and respiration, as cells without mtDNA do not have enough glutamate to convert to glutamine, a precursor for nucleotides and amino acids (Durán *et al.*, 2012). In turn, loss of these precursors would negatively influence protein synthesis. There is also proof that mitochondria are needed in the rejuvenation of the cellular environment, as it tethers to toxic protein which form aggregates in yeast, preventing aggregated protein being passed on to the daughter cell (Zhou *et al.*, 2014; Mogk and Bukau, 2014). Due to their enhanced mitochondrial function and/or their alterations to stress signalling through cofilin, mutant strains with loss of mtDNA produced more GLuc compared to the wild type. This data provides further evidence of signalling between the mitochondria and cofilin.

When comparing GLuc expression in these strains to results obtained from the killer toxin assay, there was some loss in killer toxin secretion with mtDNA removed, however this loss in rP production was less significant compared to GLuc expression. An increase in killer toxin secretion in cofilin mutant *cof1-21* (as seen with GLuc expression) was not observed, confirming that the GLuc results may be specific to the construct used. Two different signal sequences were utilised between constructs, the

K1 signal sequence for the killer toxin and the MFα1 signal sequence for producing GLuc in yeast. The results acquired using the GLuc construct may be influenced by the MFα1 signal sequence used. The findings may be specific to producing GLuc (or any other recombinant protein) with this signal sequence. It would be interesting to exchange the GLuc and Killer toxin signal sequences to confirm if the results are specific to the signal sequence used.

As there was an increase in the respiration of mutants with alterations outside of the actin binding region, this suggests other roles of cofilin as a stress sensor to the mitochondria. Microarray data from previous work in the Gourlay lab proposed that this change to mitochondria must be controlled post-translationally as levels of mitochondrial biogenesis were not apparent within the mRNA population (Kotiadis *et al.*, 2012). In light of these observations, we investigated TOR signalling by growing the strains expressing the mutant alleles of cofilin in the presence of rapamycin. We observed that strains with elevated respiration tended to show rapamycin resistance in the majority of strains (Figure 4.5). Previous microarray data of the mutant expressing allele *cof1-6* revealed upregulation of ABC transporters through ABC transporters PDR5, PDR10, PDR15 and SNQ2. This is a result of the elevation in respiratory function from the cofilin mutation, triggering a retrograde response, inducing PDR1-dependent multidrug resistance (Kotiadis *et al.*, 2012). As PDR10 along with the PDR18 and TF PDR8 were discovered to be a drug pump for rapamycin (Figure 4.9), we were able to rule out hyper-TOR signalling in strains with rapamycin resistance. This data, along with the finding that the mutant strains showed no changes in phosphorylation of Sch9 (a key target of TORC1 signalling), suggests that there was no alteration to TOR signalling (Figure 4.10).

7.3.2. MAPK signalling plays a role in the secretory pathway

Previous microarray data of cofilin mutant allele *cof1-6* from the Gourlay lab found increased levels of *TEC1* and *FUS3* mRNA compared to wild type cells (unpublished data). Both *TEC1* and *FUS3* are part of the MAPK pathway controlling filamentous growth response, yet only *Fus3* in mating pheromone response (see chapter 1 section 1.3.4 for more detail), suggesting an increase in *STE12 as this is the common TF in both MAPK pathways* (Bao *et al.*, 2004; Molina *et al.*, 2010). This signalling pathway causes an upregulation of genes involved in regulating the cell cycle, budding pattern, enhancing cellular adhesion as well as regulatory genes in elongating the cells morphology. During the filamentous response when nitrogen is limited, Ste12 binds to the filamentation response elements (FREs) as a heterodimer with Tec1 which is bound to the motif TEA/ATTS consensus sequence (TCS) (Wong Sak Hoi and Dumas, 2010) activating genes for filamentation. Conversely, during mating Ste12

binds solely to pheromone response elements (PREs), controlling the genes for proteins involved in the cell cycle, causing G1 arrest and the morphological changes needed during mating (Su *et al.*, 2010b). However, as these cells are not undergoing mating or filamentous growth, an increase in Ste12 indicates a loss of fidelity in the control of MAPK signalling. As there is no mating factor signal or nitrogen limitation, this tells us that the response is only partial as we don't see changes in cell shape. This may be due to cofilin interacting with common proteins in the pathways effecting Ste12, for example, Ras, Cdc42 or perhaps other MAPK's. Previous evidence shows that mutated cofilin leads to hyperactive Ras (Kotiadis *et al.*, 2012).

As an extension of this we tested a number of cofilin mutant alleles, and found a strong correlation between Ste12 activity, rapamycin resistance and increased mitochondrial respiration (Figure 4.12). This suggests that cofilin is involved in co-ordinating environmental sensing with mitochondrial activity and stress response. This data lead us to investigate how the removal of STE12 would influence rP production in cofilin mutant alleles. This deletion led to an accumulation of non-native GLuc detected in both the wild type and the mutants, suggesting that loss of this TF led to changes in rP processing through the secretory pathway, which is likely to be due to an increased load on chaperones in folding GLuc (Figure 5.16). These changes could be an effect of the removal of *STE12* on MAPK signalling. The signal sequence used here was different to what was used to express the killer toxin, giving a possible explain as to why we don't see the same results in both assays (Figure 4.17).

However, as we only detected moderate changes to the expression of GLuc, it is more likely that deleting STE12 prevents proper processing of GLuc within the secretory pathway. This result suggests that the control of stress response factors through Ste12 is important for the appropriate responses during rP production. Loss of this control may alter levels of proteins needed for correct folding and secretion. Future work could be undertaken to identify which Ste12 binding genes are responsible for these observed changes to rP production outside their role in regulating mating or filamentation. An interesting experiment would be to test if overexpressing protein folding chaperone would improve secretion in cells with *Δste12*. This would confirm our hypothesis of how removing Ste12 effects rP production. An example would be to overexpress the chaperone PDI, which has been shown by others to enhance rP production (Wittrup, 1995).

Figure 7.1 below portrays the interactions investigated between cofilin, Ste12 signalling and the mitochondria in stress signalling and recombinant protein production in this study with other known links.
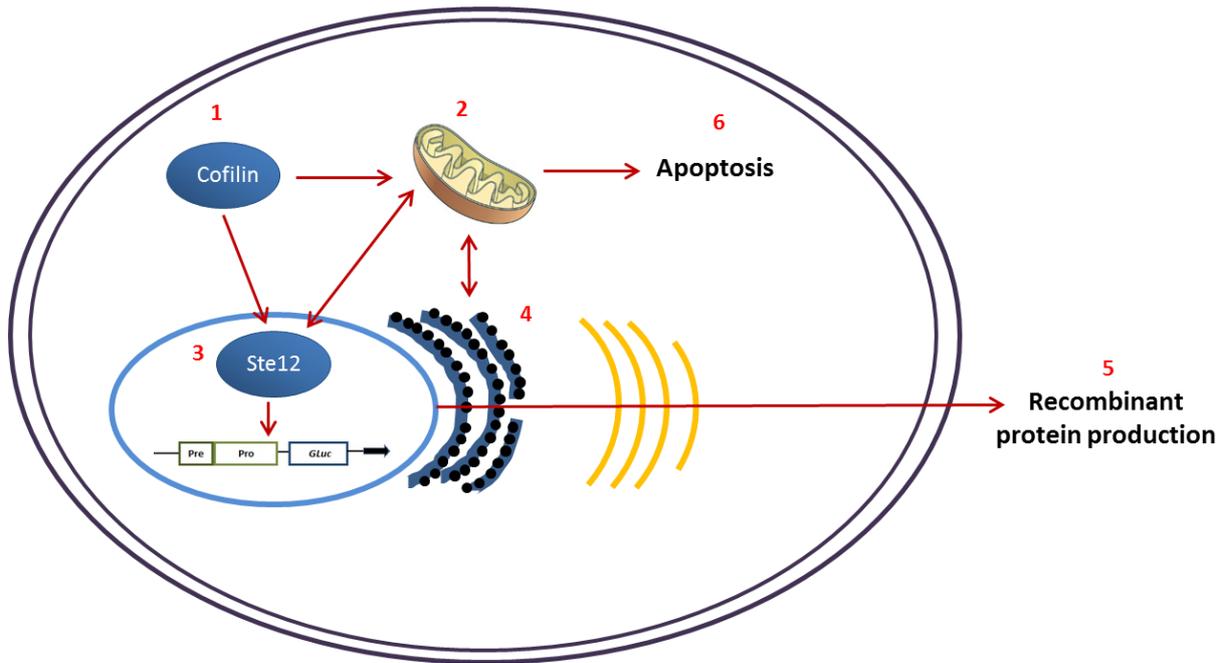
Figure 7.1 Components involved in stress signalling and its effect on recombinant protein production studied using a library of Cofilin mutant alleles. Arrows depict interactions between components investigated in stress signalling and rP production through the secretory pathway. (1) Previous data has shown that mutations to cofilin outside of its actin binding region led to alterations in mitochondrial function suggesting there is stress signalling between cofilin and the mitochondria (Kotiadis *et al.*, 2012). (2) Cofilin mutant allele *cof1-21* was the highest producer of GLuc with a significant increase in GLuc secreted. This strain and others from Class I had enhanced mitochondrial function which triggered an upregulation of drug pump activity, giving these cells resistance to rapamycin. (3) Ste12 activity across most strains also had rapamycin resistance, linking Ste12 signalling to the mitochondria during stress. It was also discovered that deleting *STE12* influences protein processing in the secretory pathway, as its deletion led to an increase in non-native GLuc. Ste12 is part of the MAPK signalling pathway in mating, therefore we believe that changes to Ste12 activity will influence GLuc expression as the signal sequence from mating alpha factor gene (MFα1) is utilised. (4) Removing mitochondrial DNA from the wild type caused an almost complete loss of GLuc produced, confirming the importance of the mitochondria in rP production. As the cross talk between the ER and the mitochondria through the ERMES junction is important in calcium signalling, this effects ER stress and mitochondrial bioenergetics possibly influencing rP production if perturbed (Kornmann and Walter, 2010). (5) rP production is heavily effected by protein processing through the secretory pathway such as PTM's and protein folding by chaperones (Walsh, 2010a; Brodsky and Skach, 2011). (6) It has been previously documented that cofilin is involved in the induction of apoptosis in the cell as it translocates to the mitochondria during oxidative stress, also linking Cofilin to stress (Chua *et al.*, 2003; Eisenberg *et al.*, 2007).

7.4 Nutrient signalling through TOR, and its impact on rP production

7.4.1 Importance of the EGO complex in rP production

TOR signalling controls many processes linked to growth and stress response and so can be described as acting as a homeostasis sensor (Laplante and Sabatini, 2012). TOR signalling is controlled by two multi-protein complexes: TORC1, which is sensitive to rapamycin controls many processes important to rP production, and the rapamycin-insensitive complex TORC2, which is best characterised for its function in cell polarity and control of the actin cytoskeleton (C De Virgilio and Loewith, 2006a; Laplante and Sabatini, 2012). Due to its role as a growth regulator, TORC1 has been identified as an important target for manipulation or control for improving rP production in higher eukaryotes (Kim *et al.*, 2012; Walsh, 2014). There is evidence that manipulation of TORC1 signalling can help improve antibody production in CHO cells when *mTOR* is overexpressed (Dreesen and Fussenegger, 2011).

The aim of the work described in Chapter 5 was to identify targets in TORC1 signalling by using the budding yeast *S.cerevisiae*, and screening strains from a TOR gene knockout library collection. The screening process involved expressing GLuc in the reporter construct as a recombinant protein. We identified Gtr1 and Gtr2 (which are part of the EGO complex in sensing amino acids) in influencing rP production. The deletion of *GTR1* and *GTR2* led to reduction in GLuc expression, implying that their function is important in rP production (Figure 5.5). Amino acid sensing in particular has been proven to control TORC1 signalling through the Rag proteins in higher eukaryotes and (homologous) Gtr proteins in yeast. This signalling controls cell growth and protein synthesis in response to amino acids available.

 In line with the deletion of *GTR1* in yeast, previous research has shown that this loss perturbs TORC1 signalling (Valbuena *et al.*, 2012). In yeast, when amino acids are added to starved cells, TOR translocates to the vacuole where the GEF Vam6 activates the GTPases Gtr1 (GTP bound) and Gtr2 (GDP bound) in a heterodimer formation (Sancak *et al.*, 2008; Oshiro *et al.*, 2014). Gtr1 signals to KOG1 (raptor in mammalian cells) directly, a component of TORC1; yet new evidence also suggests that Gtr1 may also interact directly with another TORC1 component, Tco89 (found in yeast only) (Sekiguchi *et al.*, 2014). This activates downstream processes in TOR such as ribosomal biogenesis and protein synthesis through Sch9, and other processes such as cell proliferation, mitochondrial biogenesis and stress response genes (Aronova *et al.*, 2007; Binda *et al.*, 2009; Sekiguchi *et al.*, 2014). It may be the case that by inhibiting amino acid sensing through deletion of components of the EGO complex, cells dampen TOR signalling and protein synthesis leading to a loss of GLuc expression. During starvation,

cells go into quiescent phase and autophagy is upregulated as it is no longer inhibited by TOR, recycling old proteins and organelles while sequestering nutrients for the cell to survive the starvation period (Lee *et al.*, 2007; Galdieri *et al.*, 2010; Meijer and Codogno, 2011).

Although TORC1 has a significant role in cytoplasmic signalling, it also plays a role in nuclear function such as in the regulation of the transcription of ribosomal protein gene expression through TF Fhl1, as well as mitotic cell cycle progression through Sch9 (Fingar and Blenis, 2004; Smets *et al.*, 2010). Recent evidence has also linked environmental status with the expression of pro-growth genes through modifications to histones controlling epigenetic processes (Cai *et al.*, 2011; Workman *et al.*, 2014). It has been reported that as well as an being amino acid sensor, Gtr1 and Gtr2 also play a role in negatively regulating the nuclear protein Gsp1-GTPase activity, functioning in the nucleocytoplasmic transport of proteins and nucleic acids across the membrane of the nucleus, through the nuclear protein Yrb2 (Nakashima *et al.*, 1999; Avruch and Long, 2009; Sekiguchi *et al.*, 2014). Another role for Gtr1 and Gtr2 are in their genetic interaction with INO80, a chromatin regulatory complex, proving their involvement with chromatin silencing (Sekiguchi *et al.*, 2008). Other evidence from a yeast two-hybrid assay discovered that active (GTP bound) Gtr1 interacts with the Rpc19 subunit (mammalian RPA16) of RNA polymerases I and III. Gtr1 was also found to interact with Nop8 (Nop132 in mammalian cells shares a similar sequence), suggesting a role for Gtr1 in ribosome RNA processing (Sekiguchi *et al.*, 2004; Sengottaiyan *et al.*, 2012). However, it was discovered that only Gtr1 deletion led to a reduction in RNA pol I and III activity, further proving its role in interacting with nuclear proteins (Todaka *et al.*, 2005; Wang *et al.*, 2009). These are possible mechanisms by which the overexpression of GTR1 may lead to an increase in GLuc expression due to roles outside of amino acid signalling that might be expected to influence rP production.

Interestingly, only Gtr1 and not Gtr2, can form a homodimer (Nakashima *et al.*, 1999; Valbuena *et al.*, 2012), which may explain why overexpressing *GTR1* in the wild type led to a bigger increase in GLuc produced compared to the overexpression of *GTR2* (Figure 5.5). In *Drosophila*, where the TOR pathway is very similar to mammals, overexpression of activated variants of the Rag proteins increases cell size, even when there is a lack of amino acids (Kim *et al.*, 2008; Avruch and Long, 2009). This may be due to an increase in general protein synthesis increasing GLuc production, as TORC1 stimulation increases S6K phosphorylation needed for protein synthesis. In addition, it was observed that rapamycin treatment to *Δgtr1* had an additive effect compared to the wild type, suggesting that TORC1 is still functional without Gtr1. This implies that rP production lost in the Δgtr1 mutant was through a loss of processes independent of TORC1 signalling (Figure 5.7).

As a crucial signalling pathway, TOR is highly conserved in all eukaryotes with both TORC1 and TORC2 in CHO cells(although TORC1 only has one TOR gene (*mTOR*) compared to yeast, which contains two redundant genes (*TOR1* and *TOR2*)) (Dann and Thomas, 2006). In CHO cells, amino acid sensing upstream of mTORC1 is different to yeast where there are two orthologues for each Gtr protein, RagA/B for Gtr1, and RagC/D for Gtr2. These proteins still function by activating mTOR signalling with the correct guanine nucleotide binding, activating mTORC1 (Bar-Peled *et al.*, 2012). RagA/B (GTP bound) and RagC/D (GDP bound) heterodimers recruit mTORC1 (at raptor) to Rheb, another GTPase at the lysosome when amino acids are available (Long *et al.*, 2005; Sancak *et al.*, 2008; Avruch and Long, 2009). mTORC1 also receives growth factor and energy signals from the TSC1/ TSC2 complex which negatively regulate mTORC1 by acting as a GTPase-activating protein (GAP) for Rheb. Rheb only activates TORC1 when GTP bound, although the mechanisms behind this interaction are unknown (Laplante and Sabatini, 2009; Bar-Peled *et al.*, 2012).

By utilising a CHOK1 cell line stably expressing GLuc, we examined the effects of altered mTOR signalling by transient knockdown using shRNA to measure the effects on rP production. As the yeast screen of TOR deletion strains identified that loss of amino acid sensing caused a reduction in rP production (Figure 5.5), the knockdowns of RagC and RagD (Gtr2 in yeast) were explored in CHO cells, where similar effects were observed. Although these genes were knocked down and not deleted as in yeast, any reduction in expression of the gene that was achieved led to a significant loss in GLuc expression in both RagC and RagD knockdowns (Figure 6.5). Although the qRT-PCR data did not conclusively show knockdown of these genes (Figure 6.4), repetition of RagC and RagD knockdown confirmed the loss of GLuc production in CHO cells (Figure 6.3 and 6.5), suggesting perturbation of these genes in CHO cells impacts upon recombinant protein production.

When comparing the yeast and CHO result (Figure 5.5 for yeast and figure 6.3/6.5 in CHO), there was a loss of secretion in yeast yet a loss of GLuc detected in both the supernatant and pellet in CHO cells. This suggests a difference in the effects of interfering with amino acid sensing between the single cell yeast organism and the CHO cell system. This likely reflects the differences between the regulation of amino acid sensing between yeast and mammalian cells. In mammalian cells, stimulation of TORC1 signalling requires Rheb-GTP unlike yeast cells, and even with depleted Rag protein amounts, overexpressed Rheb is able to activate mTORC1 and downstream signalling which is largely unaffected under these conditions (Long *et al.*, 2005). The regulation of the Rag proteins vary in CHO, as the Vam6 homologue Vps39 has been shown not to act as a GEF. However, it has been suggested that the protein complex Ragulator is responsible for activating the Rag proteins while functioning as an anchor to the lysosome, sharing a role with EGO proteins in yeast (Dubouloz *et al.*, 2005; Sekiguchi *et al.*, 2014).

Despite differences in their regulation, deletion of RagC/D (Gtr2) still produced the same outcome with hindered rP production in both yeast and CHO cells, confirming the importance of amino acid signalling to TORC1 and its influence on rP production.

7.4.2 Autophagy in rP production

Autophagy is negatively regulated by TOR, but is also controlled by RAS/cAMP/PKA signalling which sense nutrients in the environment (Wei *et al.*, 2008; Stephan *et al.*, 2014). The RAS/cAMP/PKA pathways influence many TOR kinases involved in glucose sensing, controlling ribosome biogenesis (via IFH1) and stress responses (via RIM15). For example, both pathways control RIM15 through its binding to 14-3-3, which controls MSN2/4 and GIS1 in stress responses to glucose starvation, causing reprogramming of the transcription machinery to change/modify cell growth and proliferation (Claudio De Virgilio and Loewith, 2006; Wei *et al.*, 2008; Galdieri *et al.*, 2010). In *Drosophila*, under nutrient starvation TOR is inhibited, as ATG1 negatively regulates S6K by blocking its phosphorylation, demonstrating cross talk between Atg1 and S6K signalling (Lee *et al.*, 2007). This strict control between autophagy and cell growth is essential, as uncontrolled autophagy is detrimental to the cell.

Autophagy is necessary for degrading organelles and protein complexes, recycling biological material needed for protein production (Levine and Klionsky, 2004). This process is also protective for the cell as it clears protein aggregates formed during ER stress, which are elevated during recombinant protein production. Yet, this process is important during normal cell growth when TOR is active as there is basal level autophagy occurring (Mizushima *et al.*, 2008). Autophagy also has a link to apoptosis through its interaction with Beclin1, with a role to prevent apoptosis by replenishing the cell with nutrients and eliminating proapoptotic stimuli (Kroemer *et al.*, 2010). Furthermore, autophagy increases lifespan as it removes damaged cellular structures such as the mitochondria. As the mitochondria plays a key role in apoptosis by the release of apoptotic factors, selective elimination of mitochondria is a cell defence mechanism (Codogno and Meijer, 2005). Autophagy therefore, is crucial to cell survival and efficient protein production by supplying the cell with recycled nutrients.

From the yeast screen undertaken in this work (Figure 5.5), we found that deleting ATG1 hindered rP production as expected due to its fundamental role in autophagy. This data demonstrated that losing this function impairs protein synthesis, as cells lose their ability to survive during starvation when nutrients become limited. Additionally, there may have been a build-up of toxic protein aggregates from ER stress causing mitochondrial collapse leading to cell death (Yorimitsu and Klionsky, 2007)(Bravo *et al.*, 2012). As autophagy is essential for an efficient secretory pathway, energy bioenergetics and cell survival, it is well conserved across CHO cells and other mammals (Figure 6.3).

Unfortunately we did not observe a positive effect on GLuc expression in yeast when overexpressing Atg1 in the wild type. This may be because Atg13 is also needed in the formation of the complex to initiate autophagy when it is no longer phosphorylated by TORC1 (Kamada *et al.*, 2010; Chen and Klionsky, 2011). If both Atg1 and Atg13 were overexpressed, this could be beneficial to the cell by improving the cells nutritional balance such as amino acids and energy via microautophagy. In addition, the energy status is also vital for cell's growth (Singh and Cuervo, 2011).

In summary, the screen performed with various TOR deletion strains in the yeast model system identified Gtr1 and Gtr2 from the Ego complex as involved in amino acid signalling. Additionally Atg1 is needed for autophagy induction, as a target for altering rP production (see Figure 7.2). Both the Ego complex and Atg1 are involved in TORC1 signalling but with opposing effects, yet both processes ensure the cell has sufficient nutrients for protein synthesis and cell growth. There are new roles emerging from the GTPases Gtr1 and Gtr2 in parallel to TOR signalling, which may account for the increase in rP production with *GTR1* overexpression.
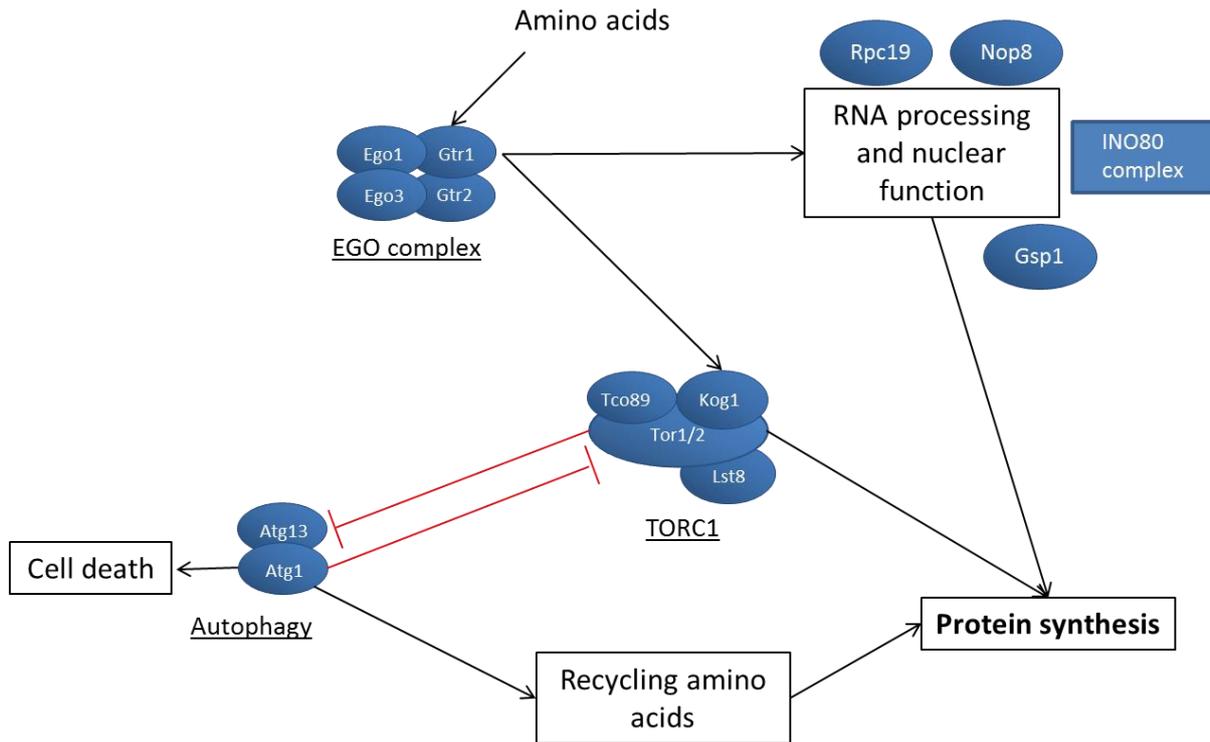
Figure 7.2 Targets identified from a screen of TOR related genes which reduce rP production when removed. Amino acids are sensed by the EGO complex (containing Gtr1 and Gtr2), activating TORC1 which induces protein synthesis downstream. Gtr1 also associates with various proteins involved in RNA processing and nuclear function, independent of amino acid signalling which may influence protein synthesis (Todaka *et al.*, 2005; Wang *et al.*, 2009). Autophagy is inhibited by TORC1 when nutrients are available by inhibiting the complex formation of Atg1 and Atg13 needed for the induction of autophagy (Kroemer *et al.*, 2010). During starvation, autophagy can also inhibit TORC1 and recycle nutrients such as amino acids in order to improve protein synthesis, or induce cell death when autophagy levels are high. Yet, there is still a basal level of autophagy needed when cells are nutrient enriched (Mizushima *et al.*, 2008).

7.5 Conclusion and future work

This research has demonstrated how both model systems investigated here, yeast and CHO cells, can be exploited for investigating stress signalling, nutrition and energy regulation as well as how this alters rP production using the model rP GLuc and killer toxin proteins. From previous work in the lab and further investigation here, we have identified that the actin binding protein cofilin plays a role in stress signalling which influences rP production, outside of its actin binding region. It was also shown that the loss of mitochondrial function effected rP production significantly. However, this loss was not as significant in cofilin mutants with mtDNA removed, confirming the signalling between cofilin and

mitochondria. The link between cofilin and MAPK signalling was confirmed by unpublished work in the Gourlay lab where cofilin mutation alleles caused high Ste12 activity with rapamycin resistance due to high mitochondrial function and upregulated drug transporters PRD10, PDR18 and TF PDR8. However, there were no changes to TOR signalling in these cofilin mutant strains as previously hypothesised (unpublished work). A new role for *STE12* was identified in protein processing, where its deletion led to an increase in incorrectly processed forms of GLuc product. This suggests that the cells have an inability to cope with the workload through the secretory pathway.

In this work, how the control of amino acid sensing through TORC1 is important through the EGO complex and basal level autophagy in dealing with the demands of rP production has been investigated. The Gtr proteins have emerging roles in the control of gene transcription and association with nuclear proteins, impacting protein synthesis parallel to their role in TOR signalling.

Below are some of the questions still to be addressed in further understanding the changes observed to rP production upon manipulation of the pathways and genes described in this thesis:

- Does increasing mitochondrial function in CHO cells enhance GLuc production? PGC1α, which is under the transcriptional control of TORC1, controls mitochondrial oxidative function which may benefit rP production. It is known that ER-mitochondrial coupling is important during ER stress as it triggers mitochondrial damage. Therefore by increasing mitochondrial function, the cell may be buffered against this stress while promoting energy supply to protein synthesis when exploiting the cell to produce heterologous protein. It is predicted that we would see enhanced expression of GLuc with PGC1α overexpression.

- Are the ERMES junctions interrupted in rho0 cells of cofilin mutants, causing loss of cross talk between the ER and mitochondria? And how does this compare between the wild type and cofilin mutants? As these junctions are important for cross talk between the mitochondria and ER, alterations may cause retardation to protein synthesis. Any alterations to the ERMES junction between the wild type and cofilin mutants perhaps seen with loss of mtDNA, may explain the significant loss off GLuc to the wild type. Perhaps cofilin co-localises to components of the junction such as MDM10, MDM15 or MDM34 which can be detected by fluorescence microscopy.

- What is causing differences in GLuc production between cofilin mutant alleles and the wild type? To develop a further understanding as to how these mutations may be affecting changes to stress signalling and rP production, the phosphorylation of cofilin can be investigated using 2D gel western blotting. As cofilin is believed to be inactivated by phosphorylation at serine 4 in yeast, alterations to this region (mutant expressing allele *cof1-4*) may alter its activity. By examining the differences in phosphorylation states, further evidence on how changes to its activity may cause alterations to rP production may be revealed.

- Is *STE12* deletion affecting the cell's capability to cope with the load imposed on the cell as a result of rP production through the secretory pathway? When deleting Ste12 there was an increase in incorrectly processed forms of GLuc in all cofilin mutant strains and the wild type. Perhaps the overexpression of a chaperone in the ER would assist the cell, for example PDI which has a role in disulphide formation would enhance the capacity of the secretory pathway in Δste12 strains. Overexpression of PDI has been shown to be beneficial in rP production (Wittrup, 1995). An increase in rP production from PDI or other enhancing chaperones, may explain the effects of removing *STE12* in the secretory pathway.

- Does the deletion of PDR10 cause rapamycin sensitivity in strains without *STE12*? We have found that rapamycin is a substrate for the multi-drug transporter PDR10, which was upregulated in the microarray analysis performed on mutant allele *cof1-6*. Therefore, it would be interesting to determine if Δste12 strains lose their resistance to rapamycin when this drug pump is removed. This information would provide further evidence towards understanding the connection between MAPK signalling and mitochondrial function.

- How would a polysome profile compare between Gtr1 and Gtr2 strains? As Gtr1 plays a bigger role in RNA processing, Δgtr1 compared to Δgtr2 may show reduced 80S monosome levels, with an increase in monosome levels with *GTR1* overexpression. An increase in monosome levels in wild type with GTR1 overexpression may increase the rate of protein synthesis, explaining why only overexpression of GTR1 enhances GLuc production. Alternatively, this overexpression may be due to increased amino acid sensing in the TOR pathway as only GTR1 can form homodimers.

The research discussed above has proven how our ability to alter cell signalling through genetic engineering in both mammalian and yeast model systems, due to their conservation between organisms, can be exploited in finding alternative ways to alter rP production. This research is also important in furthering our knowledge of the mechanisms in these complex signalling pathways, and how changes can lead to a number of diseases. For example, TOR is being investigated in cancer due to its careful control over cell growth and death.

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