College of Health and Life Sciences

Research Title

Regulation of Glucose Metabolism by p21-activated Kinases (PAKs), Ste20 and Cla4: using the budding yeast *Saccharomyces cerevisiae* as a model organism.

BY

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DEDICATION

To the creator of the entire universe, God Almighty, I dedicate this journey of my life. To my wonderful family (immediate and extended) I appreciate every one of you and dedicate the success of this work.

ACKNOWLEDGMENTS

To start with, I will like to acknowledge and appreciate my humble supervisor, Dr Thomas Hoefken for his patience, teachings and professional guidance from the start of this journey to the very end. His understanding and expertise took me from a novice to a confident researcher. Thank you, sir.

I will like to specially appreciate Dr Evgeny Makarov and Dr Sabrina Tosi, my second supervisor and RDA respectively. Your time in and out of the laboratory were of great value. Your kind advise and checking up on me to ensure I am happy and well settled helped me greatly to this point where I can say I have achieved my aim.

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To my parents and siblings, love is all I have and will always have for you. You helped me weather the storm and walked with me to see the light at the end of the tunnel.

To my angels, my beautiful wife and daughter, you are the real MVP. Love you to the moon and back. Thank you for understanding and the encouragement through the many long nights and rough days.

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ABSTRACT

Glucose, an important macromolecule, serves as a major source of energy in most organisms. Its regulation is a vital process mediated by a number of enzymes including protein kinases such as the p-21-activated kinases, PAKs. PAKs are highly conserved serine/threonine kinases that are involved in a wide range of biological functions including including glucose homeostasis. In *S.cerevisiae*, two of these kinases, Ste20 and Cla4, are well characterised. In this study, the interaction between these kinases and the enzymes involved in glycolysis, gluconeogenesis, pentose phosphate pathway and glycerol synthesis from glycolysis were tested and identified by the split ubiquitin system. furthermore, the effect of Ste20 and Cla4 were investigated in the expression level and phosphorylation of Gpd1p – an enzyme that catalyse the rate limiting step in the synthesis of glycerol- under various conditions mediated by these kinases including optimal growth condition, hyperosmotic stress and glucose response. Data obtained showed that levels of Gpd1p and its phosphorylation status were significantly reduced when both kinases are mutated but remained unchanged in single deletion strains. finally, the reduced levels of Gpd1 observed in double mutant strain of *STE20* and *CLA4* had no effect on the accumulation of glycerol. These results demonstrate that Ste20 and Cla4 contribute to protein stability of Gpd1.

Chapter 1:
Introduction and literature review

1.1 The budding yeast *Saccharomyces cerevisiae* as a model organism.

The use of model organisms in the conduction of scientific research cannot be overemphasised in present day scientific endeavour. Organisms employed as models are used as 1) they offer solutions to challenges of ethical and experimental constraints that hold for the targeted organism 2) they offer a background on which analytical methods are developed and optimized, facilitating standardisation of analysis (Hiren, et al., 2011). Cell biologists and other scientists over the years have searched other ways of carrying out scientific research on humans without the use of human subjects as specimen because humans are not experimental animals. Classic molecular genetic studies were performed on bacteria, particularly *Escherichia coli,* and they have paved the way for most of the knowledge known about this field of study. However, the challenge with the use of bacteria in understanding the concept of human biology or those of other higher eukaryotes is the evolutionary gap between the bacteria (prokaryotic) and humans (eukaryotic) (Botstein, 1991). This gap mean that bacteria and eukaryotes differ in several cellular and metabolic functions. For these reasons, scientists looked at the budding yeast *Saccharomyces cerevisiae* which has offered so many advantages.

For the sake of this work, *S. cerevisiae* will be referred to as yeast or budding yeast, unless where stated otherwise. *S. cerevisiae* is a free-living yeast commonly referred to as baker's yeast used in bread making and brewing of beer and wine. The relevance of the yeast as a model organism are numerous including, its experimental tractability similar to bacteria (such as rapid growth, genetic manipulation techniques and many others) along with close resemblance in many basic biological processes, such as metabolism (Petranovic, et al., 2010), cell cycle (Hartwell, 2002) and their role in signal transduction such as in the mitogen activated protein kinase (MAPK) pathway (Widmann, et al., 1999), to those of complex

eukaryotic cells, such as human beings . The yeast genome contains similar genes expressed in humans and all this is contained within a 12 Mbp genome making for about 1/200 the size of human genome (Botstein, 1991) and is the first to be completely sequenced. A number of studies have reported the conservation of gene function between humans and yeast (Kachroo, et al., 2015) showing that many proteins involved in biological processes have such a high degree of amino acid sequence identity in different eukaryotes and which are functionally interchangeable. Good examples are the yeast and human ubiquitin protein with 96% identicality while actin, 89% (Botstein, 1991), and the single beta and two alpha tubulins share ~75% sequence conservation with their human homologues (Luchniak, et al., 2013) . This breakthrough, has further led to findings stating that nearly 31% of all the potential protein-encoding genes of yeast have a homologue within the mammalian protein sequences. Arguably, the best example of the relevance of the yeast as a model organism is in the study of human disease genes that have been mapped by linkage, positionally cloned and then sequenced with little or nothing known about these genes except for the fact that inheritance of these gene result in a disease condition. The sequence of the gene generally provides the first clue to function by way of homology to the genes of other organisms, commonly *S. cerevisiae* (Botstein et al., 1997). Such genes include the neurofibromatosis type1 (*IRA2* in yeast) and ataxia telangiectasia (*TEL1* in yeast).

For the reasons stated above yeast has become one of the most widely employed model organisms in the study of several eukaryotic cellular processes such as metabolism (Lopez-Mirabal & Winther, 2008), regulation of gene expression (Biddick & Young, 2009), aging (Murakami & Kaeberlein, 2009), cell cycle (Nasheuer, et al., 2002) and many others. Also, study of the physiological and biochemical processes in yeast can be harnessed to

understand and possibly tackle both fungal infections in humans, animals and plants. One of such fungal infection, that poses worldwide threat to human health, is caused by the opportunistic *Candida albicans,* which is particularly harmful to individuals suffering from AIDS or generally immunocompromised individuals (Ben-Ami et al., 2008).

1.2 p21-Activated Kinases (PAKs)

p21-activated kinases (PAKs) are a group of serine-threonine kinases that are commonly known to be regulated by small GTPases such as, Rac and Cdc42, all belonging to the Rho family. Kinases are a group of enzymes that mediate their physio-biochemical role by transferring phosphate group(s) to downstream substrates in the signalling pathway they are involved in. The defining member of this kinase family, in mammals, was discovered through screening of binding targets of Rac1 and Cdc42 GTPases in rat brain (Manser , et al., 1994). These kinases were observed not to interact with the GDP bound form of Rac or Cdc42 but rather acquired a phosphotransferase activity in vitro when bound to GTP. In budding yeast, the founding member of the PAK family, Ste20, was identified through its role in the pheromone response (mating) pathway (Leberer, et al., 1992). GTPases related to Rho such as Rac and Cdc42 are known to regulate a wide spectrum of cellular processes under normal and pathological states of the cell (Knaus & Bokoch, 1998). PAKs have been implicated in mediating many known intracellular signalling processes both in human and yeast. These processes often involve the generation of distinct cytoskeletal structures (Hall, 1998) which are commonly observed during cell division, cell motility, apoptosis, cancer development etc.

1.2.1 PAKs in Yeast and Humans

In budding yeast, three members of the PAK family have been discovered, Ste20 the founding member, Cla4 and Skm1 (fig 1.1). Each of these appear to have its own distinct role with little known of the physiological role of Skm1. Ste20, as mentioned above, is the first member of the PAK family identified in yeast through its significant role in signal transduction during pheromone response in mating yeast through the activation of the

mitogen- activated protein kinase (MAPK) pathway (Mary & Jonathan, 1997). It was observed that the sterile (*ste*) mutants were unable to mate as they were unable to undergo cell cycle arrest, and neither could they change their shapes in response to exposure to mating pheromone (Lee, 2005). Other known physiological functions of Ste20 include promotion of actin polarization (Evangelista, et al., 2000), hyperosmotic stress response (O'Rourke & Herskowitz, 1998) and haploid invasive growth (filamentous growth) (Mosch, et al., 1996). Cla4, a structurally and functionally similar kinase to Ste20, was discovered during genetic screening for mutations resulting in lethality from the absence of the G1 cyclins Cln1 and Cln2 (Cvrckova, et al., 1995). This mutation led to an abnormal cytokinesis, this shows the role of Cla4 in cell division. Cla4 has been investigated and shown to be involved in the assembly of septin ring, actin polarization (Versele & Thorner, 2004) and also been shown to play a critical role in mitotic entry and exit (Hoefken & Schiebel, 2002). Studies have shown that Ste20 and Cla4 have an overlapping function as deletion of both genes renders the cell lethal.

In humans and other mammals, at least six isoforms of PAKs exist (fig 1.1) and have been reported in various tissues. They have been classed into two groups: group 1 with Pak 1, 2 and 3 which have been shown to be quite similar to yeast PAKs and the group 2 with Pak 4,5 and 6 (Baskaran, et al., 2012). Pak1 (Pakα), a protein expressed in spleen, brain and muscle (Knaus & Bokoch, 1998); Pak2 (Pakγ), a ubiquitous protein (Knaus, et al., 1995); Pak3 (Pakβ), a protein highly concentrated in the brain with a distribution difference from Pak1, with Pak1 being regionally expressed around the cortex, neurones associated with motor functions and in the cerebellum where as Pak3 was found in high expression in the region of the hypothalamus. These mammalian PAKs have similar gene sequences but differ in

function as it has been found that Pak3 dissociates from the GTPase upon activation while Pak1 and Pak2 remain associated with the GTPase (Manser , et al., 1994).

Sequence analysis studies have revealed that Pak1 is the most closely related of the mammalian PAKs to Ste20 in yeast (fig 1.1), with a divergence in sequence outside the kinase domain (Zhao & Manser, 2012).

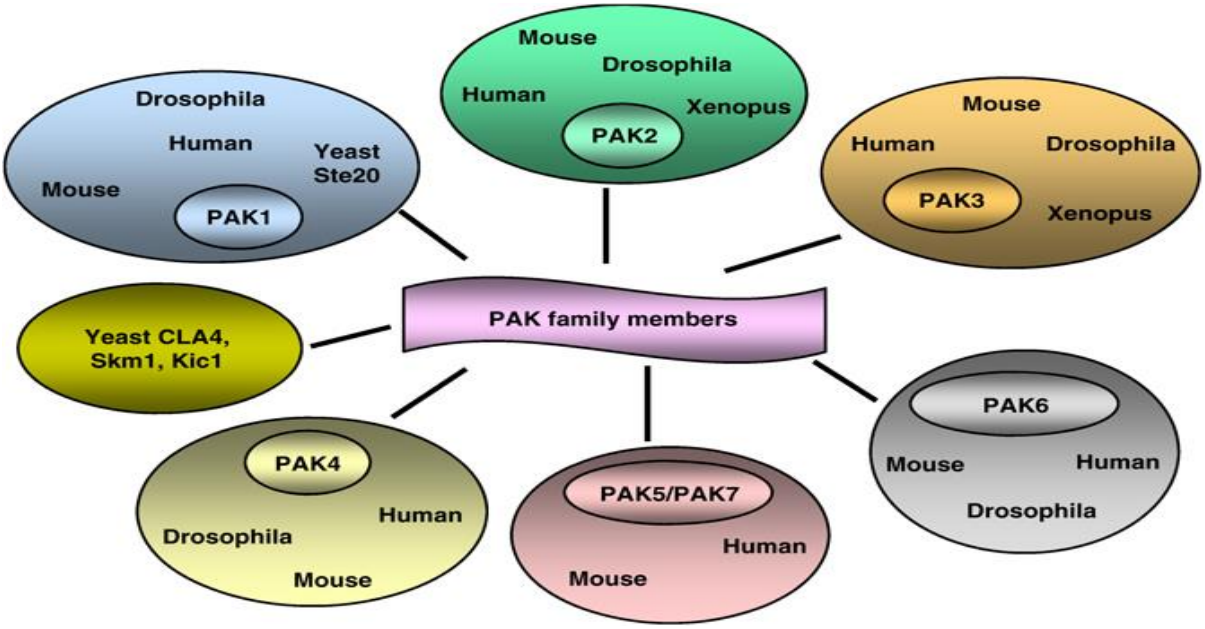


Fig 1.1. PAK family members. PAKs are evolutionary conserved in eukaryotes from the unicellular, yeast, to the complex multicellular human. Each circle shows those with structural and functional similarities, importantly, yeast Ste20 and hPAK1 as shown in the blue circle (Molli, et al., 2009).

1.2.2 Structure of PAKs

PAKs generally comprise of two domains, N- and C- terminal domains, with specific functionality (Fig. 1.2). The N-terminal domain possess a regulatory sequence characterised by conserved proline-rich motifs, approximately 60 amino-acid sequences just before the kinase domain, termed as the p21-binding domain (PBD) (Manser , et al., 1994) and Cdc42/Rac1 Interactive Domain (CRIB) (Burbelo, et al., 1995). The proline-rich motifs, PxxP

and PxP, promote the interaction of PAKs with Src-Homology (SH) domain containing proteins.

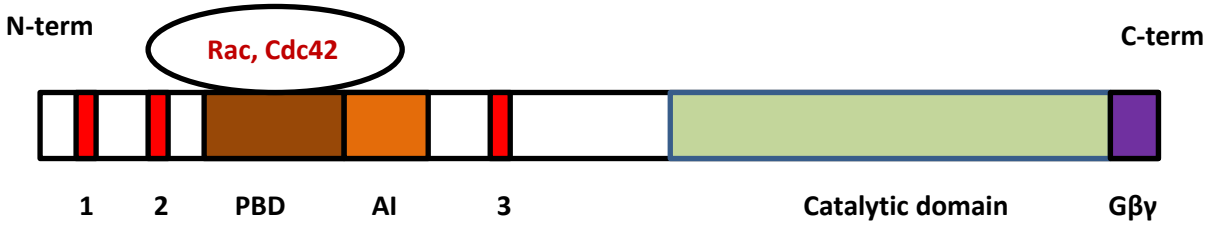


Fig 1.2. Schematic diagram showing general features of PAKs. The PBD/CRIB domain that interacts with Rac and Cdc42 (white circle) is shown in brown while overlapping to it is the auto-inhibitory domain (AI) in light brown. In red are the three-proline rich PxxP SH binding motifs. The catalytic domain is shown in light green with the overlapping Gβγ subunit binding site.

The N-terminal region is conserved across all members of the PAK except for in some with an additional Pleckstrin Homology (PH), as found in Cla4 in *S. cerevisiae.* PBD/CRIB domain confers on PAKs the ability to interact with Rac1 and Cdc42. The C-terminal domain carries the sequence that confers the catalytic kinase activity on PAKs. Studies have shown the interaction between a segment of the N-terminus and the catalytic segment of the C-terminus, causing an auto-inhibitory (AI) effect on the basal kinase activity of PAKs under unstimulated/activated conditions such as with cognate interaction with GTPase. Also, studies have shown that the association of the GTPase with PAKs induce a conformational change on the folded structure of the inhibitory domain of the N-terminus on the kinase active site, making the PAKs active for phosphorylating exogenous substrates (Leeuw, et al., 1998). Gβγ subunit is a core component of the G-protein involved in PAKs activation where its dissociation from the Gαβγ complex initiates the activation of Cdc42 characterised by the exchange of GDP with GTP (Rensing & Ruoff, 2009).

1.2.3 Activation mechanisms and localization of PAKs

PAKs are involved in many important biological signalling pathways, such as the well-studied MAPK pathway. Signalling pathways are highly complex networks involving several molecules that interact, recruit and cross talk with other molecular players of either the same or related signalling pathways; hence, requiring a strict, controlled regulatory system. Activation of PAKs is an important regulation process involving several activating molecules such as G-protein-coupled receptors, membrane-bound growth-factor receptors and integrin cell-adhesion complexes (Rakesh, et al., 2006).

Understanding the activation of PAKs from the point of G-protein-coupled receptors, signalling activates a guanine nucleotide-exchange factor (GEF) responsible for the exchange of guanine nucleotide di-phosphate (GDP) with the guanine nucleotide tri- phosphate (GTP) on the αβγ complex of the G-protein (Fig 1.3). Binding of GTP on the α-subunit of the complex stimulates the dissociation of the βγ-subunit complex – either of the free GTP- α-subunit complex and the βγ- complex can regulate several downstream effectors – including Cdc42 (Neer, 1995). The α-subunit is known to possess an intrinsic GTPase activity, which hydrolyses the GTP to GDP promoting the termination of the signalling event (Neer, 1995). Several reports have shown that the functioning and molecular mechanism of G-protein coupled signalling are conserved across all eukaryotes (Jaffe & Hall, 2005).

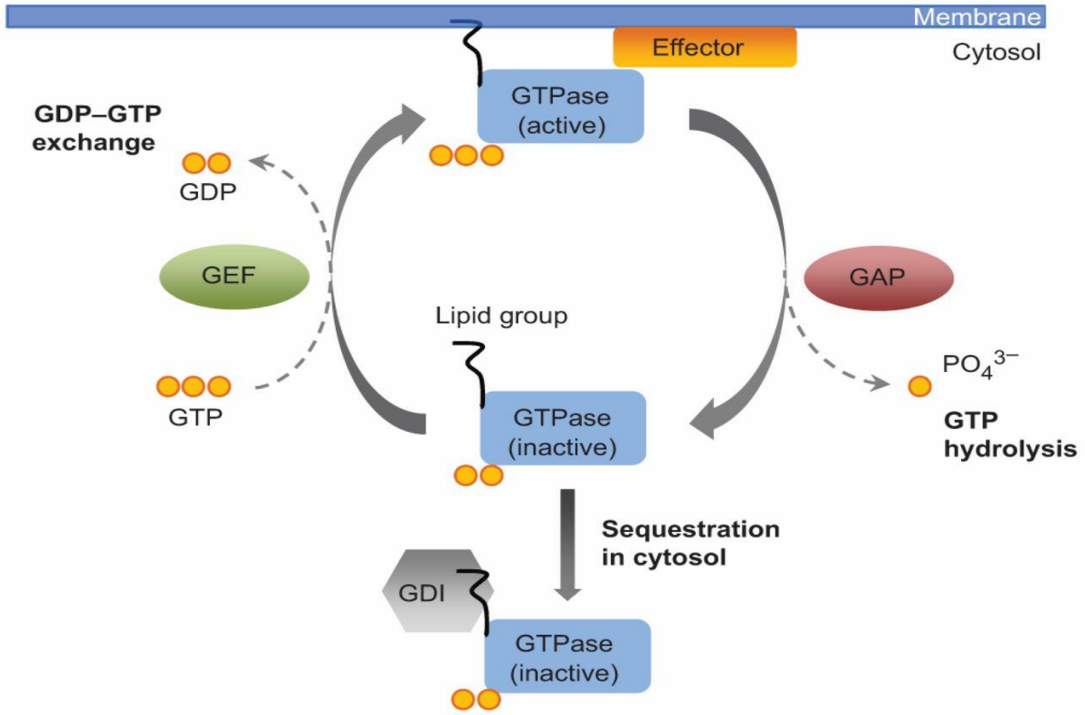


Fig 1.3. Rho GTPase cycle. The image shows a simplified illustration of the GTPase cycle with the active and inactive state represented. The interconversion of either forms of GTPase is controlled by the activating protein, guanine nucleotide exchange factor (GEF) that catalyse the exchange of GDP to GTP, guanine nucleotide dissociation inhibitor (GDI) that regulate the extraction of Rho GTPases from membranes and GTPase-activating protein (GAP) that hydrolyses GTP to GDP with the release of an inorganic phosphate group (Bento, et al., 2013).

Cdc42 becomes activated interacting with the βγ-subunit complex of G-protein coupled receptor stimulating the exchange of GDP with GTP. Binding of the Cdc42/Rac1- GTP complex to the PBD domain of PAKs disrupts the dimerization of the PAKs, resulting in a series of conformational changes that destabilizes the 3D folded structure of the PBD/CRIB/Inhibitory domain hence facilitating its dissociation from the catalytic domain (Fig 1.4) (Lei, et al., 2000). This dissociation results in the rearrangement of the catalytic domain into a kinase competent state.

Additionally, it is believed that upon activation of Cdc42/Rac GTPase, PAKs are recruited from the cytoplasm to the plasma membrane since Cdc42/Rac are both membrane

localized. Several studies have argued that GTPase interaction with PAKs alone is not sufficient enough for PAKs to attain its full phosphotransferase activity as it has been observed in other forms of PAKs, such as is observed in human PAK1 (Knaus, et al., 1995). It has also been reported in some PAKs, the dissociation of GTPase from PAKs once activation is completed (Manser , et al., 1994). Once activated, PAKs then phosphorylate multiple substrates, transducing the appropriate signal that could lead to activation of several modulating proteins and or factors. Once signal transduction is completed, GTP bound to GTPase is converted to GDP by another regulator, GTPase- activating proteins (GAPs), by hydrolysing the phosphate group on GTP. This reaction shuts off the activity of the GTPase. This model of activation of PAKs is widely supported by structural data, as well as genetic and biochemical studies (Lei, et al., 2000). Other forms of GTPase independent activation of PAKs have also been reported such as sphingosine and sphingosine-derived lipids (Bokoch, et al., 1998).

PAKs are localized in different compartments of the cell, with their presence well established in the cytoplasm where they are recruited to the plasma membrane upon activation of Cdc42/Rac. Studies have shown that they are also localized in the nucleus (Jakobi, et al., 2003) and vacuoles (Bartholomew & Hardy, 2009). Full length human Pak2 is localized in the cytoplasm but in one experiment, due to the presence of a cleavage site for caspase 3, proteolytic cleavage of Pak2 by caspase 3 in response to several apoptotic signals, generating a constitutively active Pak2p-34. Caspase 3-mediated constitutively active Pak2p-34 loses its nuclear export signal and becomes localized in the nucleus.

Ste20 and Cla4 in the budding yeast have been observed to be localized at vacuoles where they also play an important role in vacuole inheritance (Bartholomew & Hardy, 2009). They have also been reported to be found in the nucleus (Lin, et al., 2009)

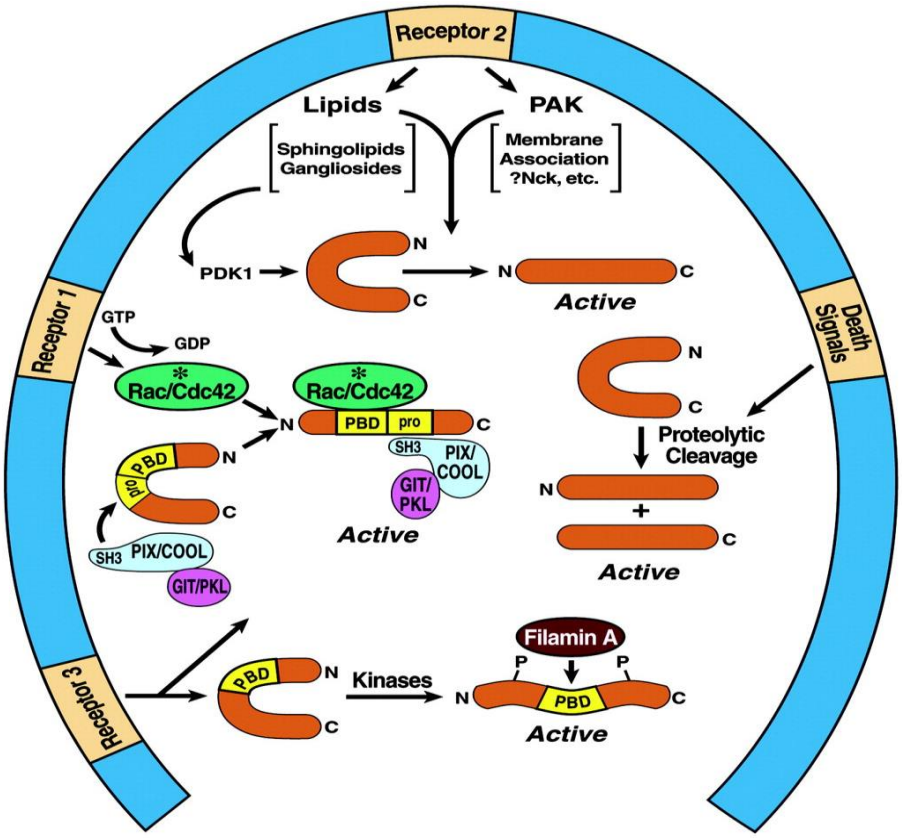


Fig 1.4. Mechanisms of PAK activation. Various mechanisms for activation of PAKs are shown based on the interacting receptor/signal. Receptor 1 shows the GTPase-dependent activation of PAKs through interaction with Rac/Cdc42. Receptor 2 shows the GTPase-independent activation of PAKs by lipid-based factors such as sphingolipids. Receptor 3 shows a kinase activation of PAKs. Finally, death signal can result in a proteolytic cleavage of the N and C terminal of the PAKs which release the auto-inhibitory effect of the N-term on the catalytic domain of the C-term (Bokoch, 2003).

1.3 Biological function of PAKs

1.3.1 Biological roles of PAKs in human

The Biology of Rho GTPases and their numerous effectors, particularly the PAKs have received a great deal of attention over the years. Numerous studies have shown PAKs involvement in a number of intricate signalling pathways that regulate a wide range of cellular activities including cytoskeletal dynamics, apoptotic signalling, cell cycle and proliferation (Bokoch, 2003) and cellular metabolism such as glucose homeostasis (Chiang, et al., 2013) as shown in fig 1.5. Also, their roles have been implicated in a number of patho-physiological conditions such as in tumorigenesis (Kumar, et al., 2006), viral infection and neuronal disease (Kelly, et al., 2013). The biologoical role of PAKs will be briefly discussed here both under normal physiological condition and pathological conditions.

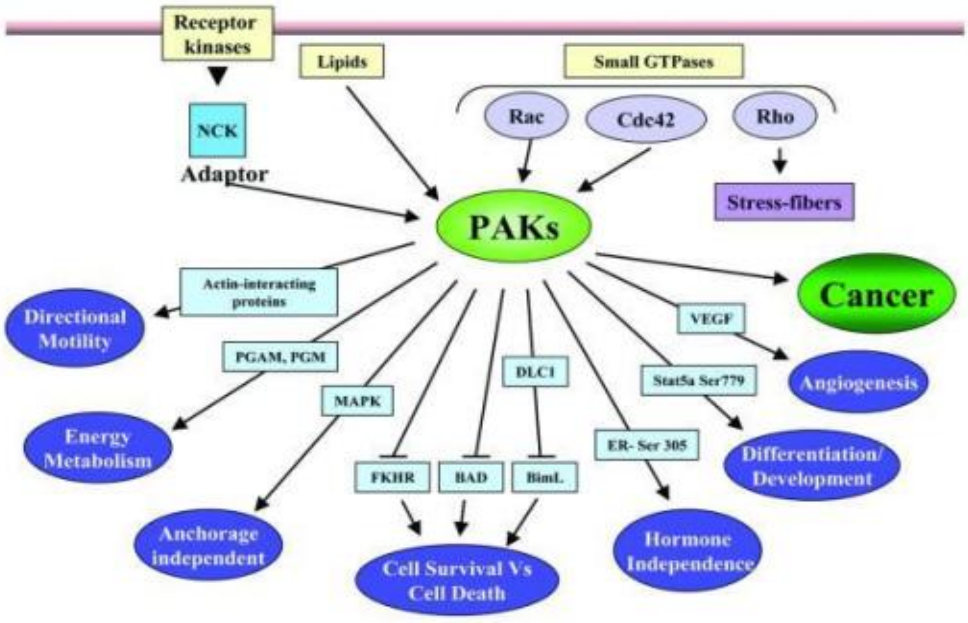


Fig 1.5 Some of the processes involving PAKs in mammalian cells. The diagram shows some interactors of PAKs and the processes they mediate. (Gururaj , et al., 2004)

1.3.1.1 PAKs and their role in cytoskeletal dynamics

The cytoskeleton - actin filament, microtubules and intermediate filaments - are an important, if not one of the most important structures of the cell. They are responsible for many activities and features within the cell such as their role in cell motility and maintaining of cell shape, cell division, organisation of cellular content and the physical and biochemical connection of the cell to the external environment (Fletcher & Mullins, 2010). Several substrates of PAKs have been identified to regulate cytoskeletal reorganisation including myosin light chain kinase, filamin A, LIM kinase (LIMK) amongst others (Dummler, et al., 2009).

Studies have shown that Pak1 activates LIMK through phosphorylation which then phosphorylates cofilin – actin binding protein – inhibiting its activity. Inhibition of cofilin has been reported to promote the integrity of actin filament network in the lamellipodium and efficiency of cell protrusion (Delorme, et al., 2007). Myosin light chain kinase (MLCK) – an enzyme that phosphorylates and activates myosin light chain which in turn regulate the activity of myosin II in non-muscle cells– has been found as a PAK substrate (Sanders , et al., 1999). Activation of myosin light chain enhances the assembly of myosin into bipolar filaments which in turn creates a tension of actin and the bundling of actin filaments into stress fibres. Paks phosphorylation of MLCK inhibits the activity of the kinase that in turn decreases the phosphorylation of myosin light chain, preventing the assemblage of the actin-myosin filament (Dummler, et al., 2009). This has led to the suggestion that PAK's ability to inhibit myosin light chain phosphorylation is responsible for the dissociation of assembled stress fibres observed in cells with overexpressed activated PAKs (Dummler, et al., 2009).

1.3.1.2 PAKs and apoptosis

Apoptosis – programmed cell death – is an important fundamental part of cell development in multicellular organism. Basically, it is a mechanism used by these organisms to rid off unwanted or dysfunctional cells in an organised fashion via controlled cellular disintegration. Apoptosis plays a critical role within the cell as it serves as a mechanism that checks and eliminates the prospect of tumorigenesis. PAKs have been shown to regulate various aspects of apoptosis and its related signalling pathway. Pak1 and 5 have been shown to exert a protective role on cells by preventing the activation of intrinsic apoptotic signals (Dummler, et al., 2009). Pak 1 and 5 stimulate the translocation of Raf1 to the mitochondria, after inducing its phosphorylation, where it phosphorylates the pro-apoptotic protein BAD and forms a complex with the proto-oncogene Bcl-2 (Wu, et al., 2008). Raf1-BAD complex and the phosphorylation of BAD prevents the binding of Bcl-2 to BAD which in turn prevents the release of pro-apoptotic factors from the mitochondria and this blocks the signal for apoptosis.

Pak2 has also been shown to possess both pro- and anti-apoptotic abilities (Dummler, et al., 2009). In the late phases of apoptosis, a constitutively active Pak2 kinase fragment has been reported to be generated from the cleaving off of the N-terminal regulatory domain of Pak2 by caspase-3, which promotes cell daeth (Rudel & Bokoch, 1997).

1.3.1.3 PAKs and cell cycle

PAKs have been reported to play critical roles during cell cycle progression with studies identifying the kinase activity to be at its peak at mitotic entry and a sustained during mitotic progression (Dummler, et al., 2009). Localization of Pak1 to specific structures

involved in and during mitosis including mitotic spindles, chromosomes and contraction during cytokinesis (Li, et al., 2002).

Tubulin Cofactor B (TCoB), a cofactor involved in the assembly of alpha/beta tubulin, has been reported as a substrate of Pak1, where Pak1 phosphorylates and colocalizes TCoB on newly ploymerised microtubules and centrosomes (Vadlamudi, et al., 2005). Microtubule dynamics has been inextricably linked to mitotic spindle function and the dysregulation of Pak1 and TCoB has been shown to result in a multiple spindle phenotype. Also, Pak1 has been suggested to play a role in chromosome condensation in the early stages of mitosis. Pak1 has been reported to associate with Histone H3, colocalizing and phosphorylating it bringing about the initiation of chromosomal condensation (Li, et al., 2002).

1.3.1.4 PAKs and human MAPK

In humans, PAKs have been implicated in the regulation of MAPK signalling pathway with keen attention to those involved in response to environmental stress and cell proliferation. Paks have been shown to modulate the activity of c-Jun amino terminal kinase (JNK) and p38 MAPK cascades, important kinases in the response to environmental stresses (Knaus & Bokoch, 1998). p38 has an equivalent homologue in the budding yeast, Hog1. Both Hog1 and p38 mediate the regulation of cells from osmotic stress with p38 also showed to be involved in inflammatory response (Han, et al., 1994). The effect of Pak in the p38 MAPK cascade has been identified in the phosphorylation of upstreams components of this pathway.

1.3.1.5 PAKs and the regulation of glucose homeostasis

Beyond their roles in non metabolic processes, PAKs also mediate important molecular processes such as glucose homeostasis. The role of Pak1 has been identified in the uptake of glucose in skeletal muscle, secretion of insulin and in the production and function of incretin hormone, GLP-1 in the intestine (Chiang & Jin, 2014). Rac1/Pak1 has been shown to play a fundamental role in the regulation of insulin-stimulated GLUT-4 vesicle mobilization with a deficiency of either also shown to result in an impaired uptake of glucose by the muscle (Chiang & Jin, 2014). Activation of Cdc42-Rac1-Pak1 in the pancreatic β cell cascade responsible for the secretion of insulin in response to elevated plasma glucose has been reported (Wang, et al., 2007). The role of Pak1 in this cascade has been demonstrated in mouse and human where depletion of Cdc42 and Pak1 prevented the second phase of glucose-stimulated insulin secretion (Wang, et al., 2007).

In a separate study, Pak1 was shown to inhibit phosphoglycerate mutase, an enzyme of the glycolytic pathway responsible for the interconversion of 3- and 2-phosphoglycerate, hence switching from glycolysis to PPP with the increased generation of NADPH and in neutrophils, this leads to an increased oxidative and microbial phagocyte response (Shalom-Barak & Knaus, 2002).

1.3.2 Biological roles of PAKs in yeast

1.3.2.1 PAKs and MAPK pathways in yeast

Signal transduction is a fundamental property of living cells to changes in their environment. One of the well utilized channel for eliciting these environmental responses is the MAPK pathway (Widmann, et al., 1999). Most of what is known of MAPK pathway is based on extensive research first conducted in *S. cerevisiae* and studies have shown components and mechanisms by which they operate have been conserved down the evolution lineage of the eukaryotic kingdom (Chen & Thorner, 2007).

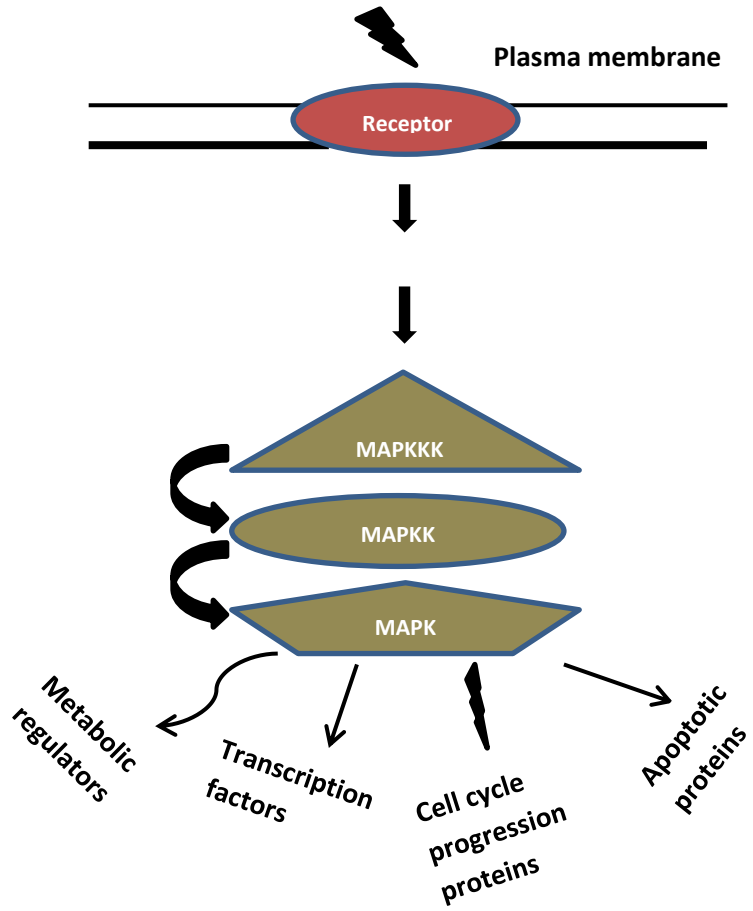


Fig 1.6. Simplified representation of MAPK pathways in budding yeast. Extracellular signals are transmitted through plasma membrane-borne receptors which recruits appropriate effector molecules to the membrane, initiating a cascade of events resulting in the activation of MAPKKK, MAPKK

A brief overview of the MAPK cascade shown in fig 1.6 will be described and then proceed to describing three key pathways in yeast regulating responses to pheromone , nutrient

deprivation (filamentation) and solute/ion imbalance (HOG pathway). The canonical MAPK pathway comprises of three key kinases, a MAPK kinase kinase (MAPKKK or MEKK) activates a MAPK kinase (MAPKK or MEK), which then activates a MAPK (or ERK, extracellular signal-regulated kinase in mammals) (Chen & Thorner, 2007). Once activated, MAPKKKs activates MAPKKs by phosphorylating at least two serine or threonine residues at conserved motifs - Thr- X- Tyr- within the activation region. Activated MAPKK then activates its target MAPK by phosphorylating the threonine and tyrosine residues of a conserved –Thr- X- Tyr motif within the activation region (Chen & Thorner, 2007). Activated MAPKs then regulate a wide spectrum of cellular activities by phosphorylating a diverse set of substrates such as phosphatases, metabolic regulators, transcription factors, translational regulators and proteins involved in cell cycle progression, gene expression amongst others (Chen & Thorner, 2007). However, two more MAPK pathways exist in budding yeast not regulated by PAKs.

1.3.2.1.1 Pheromone response pathway

In budding yeast, mating is an important process regulated by specialized mating factors, pheromones (Hao, et al., 2007). Mating occurs, between two different haploid cell types marked in the budding yeast by the *MAT* locus. The *Mat* locus is defined by either the **a** or α region and those with *Mat* **a** locus is termed as **a** cells while those with *Mat* α locus are termed α cells (Heitman, 2007). Each cell type secrete pheromones or sex factor after its kind i.e α cells secrete α factor while **a** cells secrete a factor. The pheromone response pathway has been well studied and reports have shown that Ste20 plays a vital role in this signalling pathway (Ludger & Peter, 2009). This finding was uncovered from genetic

screening of suppressors of mating defects indicting the essentiality of Ste20 in mating in yeast (Hofmann, et al., 2004).

The pheromone response pathway is initiated by the interaction of the a or α factor and its cognate G-protein coupled receptors, GPCR, Ste3 or Ste2 respectively as shown in Fig 1.7. Associated with the GPCR is the heterotrimeric G-protein complex comprising of the αβγ subunits that act in the exchange between GDP and GTP hence acting as a guanine nucleotide exchange factor (GEF) (Chen & Thorner, 2007).

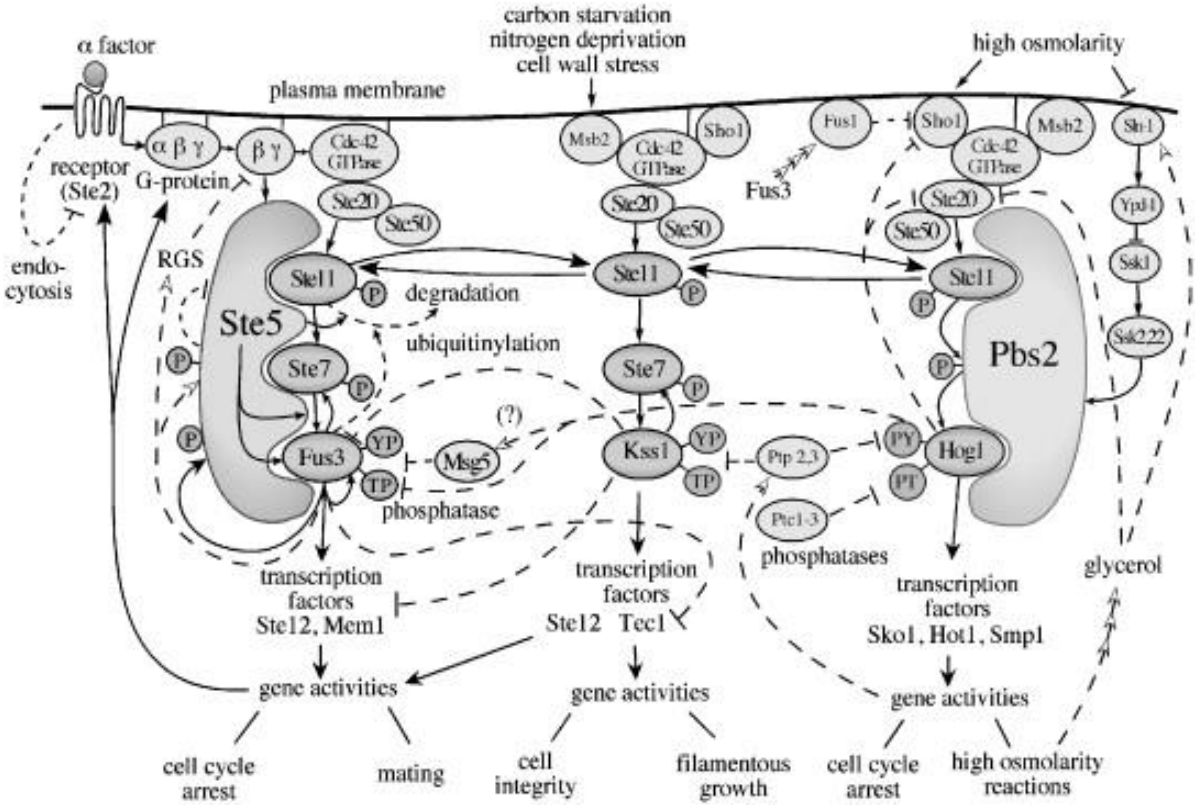


Fig 1.7. Detailed MAPK pathways in yeast. This shows three of the well studied MAPK signalling, to the left is the pheromone response pathway, the middle is the invasive filamentous (or nutrient deprivation pathway) and the right is the high osmolarity glycerol pathway (Rensing & Ruoff, 2009).

Activation of the complex results in the dissociation of the Gα-subunit (Gpa1) from the Gβγ complex (Ste4-Ste18 complex), with the Gβγ subunit recruiting other key effector proteins

of the pheromone response pathway: Ste20, Ste5 adapter protein (Ostrander & Gorman, 1999) and Far1 (O'Rourke & Herskowitz, 1998) towards the membrane. Far1 recruits and activates Cdc24, the GEF of Cdc42, by binding to the N-terminal RING-H2 domain of Gβγ subunit. Activated Cdc42: Cdc42-GTP, then activates Ste20 and or Cla4 by disrupting the autoinhibitory effect of the CRIB domain on the catalytic domain. Cdc42 interaction with Ste20 has been shown to promote the localization of Ste20 to sites of polarisation and subsequent stabilisation of Ste20 (Gagiano, et al., 2002).

Reports of the expression of a Ste20 mutant that cannot bind to Cdc42 have shown an impairment in the response to pheromone signaling in yeast (Mosch, et al., 1996). Ste20 activates three different MAPK pathways: pheromone response, high osmolarity glycerol and filamentous growth pathways. It activates all of these via phosphorylation of Ste11, a MAPKKK hence serving as a MAPKKKK. Cla4, a structurally and functionally related PAKs to Ste20 (Keniry & Sprague Jr., 2003), has also been shown to interact with Cdc42 and form a part of a multicomponent complex that activate the MAPK pathway by phosphorylating and activating Ste11 (Hofmann, et al., 2004). Ste11 further phosphorylates and activates another important MAPKK protein Ste7 that bring about the complete transduction of the pheromone response, Ste7 (MAPKK) phosphorylates Fus3 (MAPK) (Jones Jr. & Bennett, 2011). All three pheromone kinases : Ste11, Ste7 and Fus3, are held in close proximity by the scaffold protein Ste5 (Choi, et al., 1994) promoting the local concerntration and ease of activation of the pheromone response.

The pheromone response MAPK, Fus3, activates a number of genes including the transcription factors Ste12 and Mem1. The transcription factor Ste12 activated is involved in both pheromone response and filamentous growth response but . In response to

pheromone, Ste12 directs the expression of a number of mating-directed genes through Ste12 binding sites or pheromone response elements, PRE, on these genes (Chen & Thorner, 2007; Sprague & Thorner, 1992). Studies however, have shown that pheromone stimulation results in the transient activation of another MAPK, Kss1 ,an important kinase of the filamentous growth response (Ma, et al., 1995). Kss1 activation is dependent on the kinase activity of Ste11 and Ste7 similar to Fus3 activation process but independent of the Ste5 scaffold protein (Flatauer, et al., 2005). Deletion of both Fus3 and Kss1 results in sterility of such cells, while one of either alone is enough to elicit the mating process – this indicates the redundancy of both proteins. However, studies have shown that the loss of Kss1 has little effect on mating proficiency compared to the loss of Fus3; with an efficiency reduction of about 10% of the wild type level (Ma, et al., 1995).

1.3.2.1.2 Filamentous growth pathway

Filamentation is an interesting as well as an important aspect of fungal growth and life cycle. Filamentous growth study is a major area of concern as it is one form of growth involved in pathogensis and virulence (Paul & George, 2012). Fungal pathogenesis poses a huge threat to human lives, as seen in the case of the opportunistic commensal human fungi, *C. albicans,* that infect its host when they become immunocompromised from infections andplant lives, as is the case of the destructive plant fungi, *Magnaporthe oryzae* that leads to massive loss of crop yield and quality of crops becoming a threat to human health and the global economy (Yang, et al., 2017).

Filamentous growth has been described as a fungal differentiation behaviour in response to external stumuli usually nutrient limitation (Cullen & Sprague Jr., 2012). Response to filamentation varies within different species of fungi and they range from hyphae formation

to mycelial mat formation (Cullen& Sprague Jr., 2012). Studies have shown many species of fungi undergo this growth cycle including budding yeast, , human (e.g *C. albicans*) and plant (*M. oryzae*) pathogens. Most studies and our current understanding of the regulation of this process has been carried out on the budding yeast *S. cerevisiae* (Paul & George, 2012) as a result of its amenabilty to various genetic techniques (Botstein & Fink, 2011).

S. cerevisiae does not undergo true hyphae formation in response to filamentous stimulus, instead they adopt a pseudohyphal pattern of growth in diploid cells and an invasive growth in haploid cells (Chen & Thorner, 2007). These two cell types, haploid and diploid, however share similar signalling pathway in a number of cellular processes including filamentation. Worthy of mention is the fact that filamentation only occurs when cells grow on solid medium unlike true hyphal growth, pseudohyphae undergoes cytokinesis, they are not multinucleated and remain attached to each other by cell wall proteins. However most fungi, exhibit similar life cycle consisting of the yeast-form and filamentous-form (Cullen and Sprague Jr., 2012). Three key changes define the process of filamentous growth in yeast; cell-cell adhesion, a reorganisation of cell polarity and a pronounced increase in cell length. Different studies have supported the fact that nutrient limitation is one of the key stimulus regulating the filamentous process according to works done by Gimeno et al, 1992; nitrogen level and filamentation and Cullen and Sprague 2000 fermentable carbon and filamentation.

Several signalling pathways regulating filamentation in yeast have been identified but here, our attention is towards the MAPK pathway. Many of the molecular and signalling components involved in the filamentous growth response are the same for both pseudohyphal growth in diploid cells and invasive growth in haploid cells (Truckses, et al., 2004). Several studies have shown that many of the core components of the mating/

pheromone response signalling are implicated in the signalling leading to filamentous growth in yeast (Liu, et al., 1993). One of the key proteins of this pathway is Ste20, our protein of interest. Others include Ste11, Ste7, Kss1 and the transcription factor Ste12 as shown in fig 1.7. Cdc42 and its cognate GEF, Cdc24, have both been implicated in the regulation of the filamentous growth.

Upon receiving an adequate stimulus of depleted nutrients, two key transmembrane proteins are activated, Msb2 and Sho1, which are found upstream of the filamentous growth response pathway (Cullen, et al., 2004). Cdc42 is found downstream of the signalling route from Msb2 and Sho1. The relevance of Cdc42 and its associated GEF, Cdc24, has been indicated in their involvement in controlling cell polarisation in *S. cerevisiae* as studies on strain carrying a temperature sensitive allele of Cdc42 showed blocked bud formation at the temperature of restriction (Zhang, et al., 1999). Cdc42 has been shown to associate with Ste20 and Cla4 (Gagiano, et al., 2002). Ste20/Cla4 activates the MAPKKK Ste11. In filamentous growth pathway, the main function of Ste11 is the phosphorylation of the MAPK kinase, Ste7 – also the MAPK kinase of the pheromone response pathway. The difference however lies in a second adaptor molecule, Ste5 - which is involved in the pheromone response pathway - and not required for filamentous growth signalling. Ste7 functionally activates and phosphorylates Kss1, a MAPK, responsible for the complete transmission of filamentous growth signal activating downstream transcription factor such as Ste12 (Gustin, et al., 1998). Ste12 is a common transcription factor involved in both pheromone response pathway and filamentous growth pathway. Studies have shown that Ste12 interacts with two other transcription factors, Tec1 and Mcm1, in activating a large number of filamentous growth and pheromone response pathways respectively.

1.3.2.1.3 High osmolarity glycerol pathway

When cells are exposed to a higher solute concentration from extracellular medium in which they exist than those of the intracellular medium, there is a rapid drop in tugor pressure which can be lethal to cell survival if no mechanism is in place to create an osmotic balance. In the event of such osmotic imbalance, *S. cerevisiae* has developed an internal mechanism by increasing the concentration of a soluble and inert osmolyte, glycerol (Chen & Thorner, 2007). This mechanism involves the activation of a MAPK pathway referred to as the High Osmolarity Glycerol (HOG) response. The key activator of this pathway is the Hog1, with its functional ortholog in mammalian cells known as p38 – a stress-activated MAPK (SAPK) (Han, et al., 1994).

In *S. cerevisiae,* the activation of HOG response to osmotic stress exist via two distinct routes (Fig. 1.7). The first route involves phospho- transfer between histidine-aspartate module which exist between the first transmembrane osmosensor, Sln1. When stressor signal is received, Sln1 becomes inhibited as it becomes dephosphorylated at its histidine residue which is autophosphorylated under iso-osmotic condition. This allows for Sln1 to associate with and increase the amount of unphosphorylated Ssk1 which binds and activate Ssk2/Ssk22 complex, a MAPKKK. Ssk2/Ssk22 phosphorylates and activate a MAPKK of the HOG pathway, Pbs2, which then activates Hog1 (Posas & Saito, 1998).

A second route, which is of interest to this study, is activated via Ste20 and Cla4. Here, the osmo-sensor Sho1 serves as the membrane signal receptor. Sho1, a transmembrane protein receptor comprises of four transmembrane domains near its N-terminus and a cytoplasmic SH3 domain as reported by Desmond, et al., 2000. Sho1 also responds to osmotic stress via interaction with two putative osmosensors, Msb2 and Hkr1 (Tatebayashi, et al., 2007). Sho1

physically interacts with the important MAPKK osmoadpator, Pbs2, via its SH3 domain (Willem & Marco, 2002). This interaction has been reported to suggest that Sho1 SH3 domain has a docking site for other important osmosensing proteins involved in the HOG pathway such as Hkr1 (Raitt, et al., 2000). Docked to Pbs2 are Cdc42, Ste20, Ste50 and Ste11 (Reiser, et al., 2000). Cdc42 GTPase interacts with Sho1 further assisting the recruiting of Pbs2 to the site around the cell wall/membrane believed to be most susceptible to osmotic stress such as the bud emergence site (Reiser, et al., 2000). Ste20 is recruited towards the cell membrane via Cdc42 GTPase. Important to stress here is that Ste20 is also recruited by the Sho1-directed osmosensors Msb2, via the scaffold protein Bem1 and actin cytoskeleton, and Hkr1 which binds to the SH3 domain of Sho1 (Tatebayashi, et al., 2007). Bound Bem1 to the cytoplasmic domain of Msb2 initiates signal that leads to the recruitment of Ste20.

Ste20 is believed to be co-activated by Ste50, which has been suggested to be a co- factor for Ste11 (O'Rourke, et al., 2002), a MAPKKK also involved in the pheromone and filamentation pathway. Ste20 phosphorylates and activates Ste11 thus, acting as a MAPKKK Kinase. Phosphorylation of Ste11 by Ste20 causes the dissociation of the inhibitory N-terminal from the catalytic C-terminal domain. Due to the involvement of Ste11 in the pheromone response and filamentous pathway, it has been argued that there must be a way the yeast cell has developed a faithful maneuvering system such that Ste11 does not encounter any of the activators involved in the pheromone response and filamentous pathway, i.e Ste5 and Ste7 respectively. Three of the hypothesised maneuvering systems are (1) Ste11 interacts by binding dirctly to Sho1 via the cytosolic tail of its C-terminal (Zarrinpar, et al., 2004), (2) Ste11 binds tightly to the MAPKK, Pbs2 which is downstream of the Hog-MAPK pathway (Posas & Saito, 1997), (3) Ste11 requires the non-catalytic binding

of Ste50, believed to be a subunit of Ste11 (Kwan, et al., 2006), which can associate with both a membrane-anchored protein, Cdc42 (Truckses, et al., 2004) and an integral membrane protein, Opy2 (Wu, et al., 2006). Ste11 phosphorylates Pbs2, as mentioned above, which in turn phosphorylates and activate Hog1. Activation of Hog1 results in the translocation to the nucleus from the cytoplasm where it binds and phosphorylates several transcription factors as well as regulate the expression of several genes in response to the hyperosmotic shock (de Nadal, et al., 2002).

1.3.2.2 Regulation of MAPK signal pathways

1.3.2.2.1 Regulatory strength of MAPK scaffolds

The MAPK pathway depends and consist of several proteins that serve as adaptor, scaffold and/or anchoring role in ensuring the correct signal protein is engaged upstream at the plasma membrane and at the same time recruiting the needed effector protein downstream of the signal pathway. One can say, the importance of such scaffold proteins is to ensure the minimization of the risk of activating an inappropriate or unproductive responses.

In the hyperosmotic stress response, Pbs2 serves as a scaffold/adaptor for the HOG pathway binding several integral membrane proteins of the Sho1 osmosensor route including Ste11 and Hog1 as well as the dedicated MAPKK of the same pathway (Chen & Thorner, 2007), Fig. 1.7. Pbs2 also serve as the MAPKK of the Sln1 pathway as it interacts with the MAPKKKs, Ssk2 and Ssk22, hence a central regulator of the HOG pathway. Mutation of the site of phosphorylation in Pbs2 (Ser514 and Thr518) leads to osmosensitivity of cells while mutation to aspartic acid results in the constitutive activation of the HOG pathway (Wurgler-Murphy, et al., 1997). Ste5, a known effector of the pheromone response pathway

was one of the first MAPK scaffold protein identified in any organism and also the best characterized (Elion, 2001, Dard & Peter, 2006). Ste5 has been found to exist as a monomer which can self-associate to form an oligomer which then activate the pheromone response by presenting docking sites for the recruitment and binding of Ste11 allowing for interaction with the PAK Ste20 (Chen & Thorner, 2007). Ste5 also provide docking sites for the interaction of Ste7 and Fus3. This specific interaction of Ste7 and Fus3 conditions the received signal to activate the pheromeone response pathway and not the filamentation, as both pathway share similar modules in Ste7 and Ste11. Another regulatory role of Ste5 is seen in its recruitment to the membrane by the key pheromone receptor Ste2/Ste3 allowing it to bind Gβγ subunit of the G-protein complex, Gαβγ (Choi, et al., 1994). The interaction of Ste5 with Gβγ subunit commits Ste11 and Ste7 to the pheromone response pathway as both are involved in other MAPK pathways.

1.3.2.2.2 Protein inactivation and degradation

One of the key component of every signalling process in a cell is the accumulation and activation of proteins and other factors involved in such processes. Some signals are capable of affecting more than one process within a cell, for instance, response of cells to hyperosmotic stress condition affect both developmental transitions and viability of cells as such it is necessary for a check and balance system to be set up by the cell to ensure that responsive proteins are tightly regulated. A specific example is the Fus3 and Hog1 MAPKs responsible for the pheromone response pathway and high osmolarity glycerol pathway respectively, both of which are known to induce cell cycle arrest, are known to be inactivated within an hour or less after their activation permitting cells to resume vegetative growth (Chen & Thorner, 2007). One of the known mechanism of activation of MAPK

proteins is via phosphorylation of both the Thr and Tyr in their activation loop making the dephosphosrylation by protein phosphatases an effective route of inactivating these proteins in the absence of suitable activating signal or after effecting the required physiological process. Fus3 and Hog1 are known to be deactivated by the protein-tyrosine phosphatases Ptp2 and Ptp3 while Fus3 has also been shown to be inactivated by the dual-specificity phosphatase Msg5 and Hog1 by the serine- threonine phosphatases Ptc1,Ptc2 and Ptc3 (Martin, et al., 2005)(Fig. 1.7).

Another known path of deactivation of proteins is the timely mannered ubiquitin-mediated degradation of pathway component proteins. For example, in the pheromone response pathway, degradation of Ste7 plays a crucial role in the recovery from pheromone-induced cell cycle arrest (Wang & Dohlman, 2002). This activity-induced degradation of Ste7 is dependent on its phosphorylation by activated Ste11. Another noticeable aspect of this activity-induced degradation is the stimulus-induced degradation of Ste11. Ste11 degradation has been reported to be favoured upon tethering with Ste5 than it is with Sho1, Pbs2 and Ste50 (Esch & Errede, 2002).

1.3.2.2.3 Specific protein-protein interactions

In the transduction of signals, regulation of target of choice is an essential and carefully executed process. One of the simplest mechanism employed by cells is the differential binding or specificity of interactions such as is observed with enzyme-substrate relationship. Using as an example, Fus3 and Kss1 are both activated during pheromone response signalling. However, Fus3, and not Kss1, serves as the principle mediator of the associated cell cycle arrest because it best phosphorylates Far1, an inhibitor of the cell cycle protein Cdk (Breitkreutz, et al., 2001), and studies have shown that this preferential selectivity is not

down to the differences in catalytic efficiencies or activities of these proteins but rather due to the presence of a docking site on Far1 that interacts with and recruits Fus3 but not Kss1 (Remenyi, et al., 2005). This is believed to be one of the mechanism employed by cells to discriminate against similar acting enzymes or proteins such as Fus3 and Kss1, thus authorizing Fus3 to bring about cell cycle arrest upon pheromone stimulation and blocking any potential Kss1 induced arrest which is needed during nutrient depletion in cells.

Another mechanism commonly employed by cells is the specific binding of proteins to distinguish among an array of similar proteins (Chen & Thorner, 2007). This type of binding specificity is well observed among activator-scaffold and scaffold-kinase, a unique example is obsereved in the HOG pathway where SH3 domain of the osmosensor Sho1 specifically binds to the proline-rich motif on the scaffold protein, Pbs2 (Marles, et al., 2004). The specific binding of Pbs2 by the SH3 domain of Sho1 enables the HOG pathway signalling, whereas mutation about this domain that increases the strength of this interaction has been reported to increase the resulting signal (Marles, et al., 2004). It has been reported that Pbs2 motif has the ability to bind SH3 domains in other organisms but in the yeast, it only binds to the SH3 domain of Sho1 making it specific for the activation of the HOG pathway (Zarrinpar, et al., 2003). It has also been shown that mutation leading to the decrease in interaction between Sho1 and Pbs2 allowed for the increase in osmostress induced cross talk to the pheromone response pathway (Marles, et al., 2004).

Another classic example is seen at the level of MAPKKKs, speaking specifically of Ste11. In the MAPK pathway, Ste11 is involved in the activation of several different MAPKKs such as Ste7 (in both pheromone response and filamentous growth response) and Pbs2 (HOG pathway). The particular signalling pathway activated by Ste11 is dependent on the nature

of scaffold protein it interacts with, for example, signalling occurs only through the pheromone response pathway when Ste11 binds to Ste5 while in the absence of Ste5 scaffold, signalling occurs through the filamentous growth pathway but when it binds to Pbs2 then it switches signalling through the HOG pathway (Harris, et al., 2001). Also looking at this specific protein-protein interaction at the level of MAPKKs and MAPKs. Ste7, a MAPKK common to both pheromone response and filamentous growth pathway, activates MAPKs of the two pathways mentioned which are Fus3 and Kss1 respectively. For the activation of Fus3 by Ste7, associative interaction with Ste5 is required but in the absence of such interaction, ste7 avtivates the more preferred Kss1 (Maleri, et al., 2004).

1.3.2.3 PAKs and their role in mitosis

In yeast, Ste20 and Cla4 have been implicated in mitosis in different capacities. In one study by Hoefken and Schiebel (2002), Ste20 and Cla4 were shown to play different roles in mitotic exit. Mitotic exit network in yeast is a signal transduction cascade that controls the exit of mitosis and prepares the cell for the commencement of the next cell cycle (Hofken & Schiebel, 2002). Cla4 was shown to be involved, via a Cdc24-Cdc42-Cla4 pathway, in the direct localization of Lte1, a key component of the mitotic exit network, to the the site of developed bud cortex enabling it to bind.

The exact role of Ste20 in mitotic exit only points towards the possiblity of it having an overlapping role to Lte1. Unlike Cla4, Ste20 is not required for the activation nor the localization of Lte1 to the bud cortex (Hoefken & Schiebel, 2002). An allele of Cdc42, *Cdc42-118,* was shown to target Lte1 towards the bud cortex but cannot survive without the presence of Ste20. Furthermore, *Δlte1Δste20* has been shown to be lethal due to a defect in regulation of mitotic exit but not *Δlte1Δcla4.*

1.3.2.4 Role of PAKs in sterol homeostasis

Sterols belonging to the lipid family serve numerous functional roles in eukaryotes. They form lipid components of eukaryotic cell membrane and also characterise these membranes (Lin, et al., 2009). In budding yeast, it has been reported of their important role in cell polarity particularly during mating (Tiedje, et al., 2007). Ste20 has been shown to bind several enzymes, Erg4, Cbr1, Ncp1 involved in sterol biosynthesis (Tiedje, et al., 2007). Ste20 and Cla4 have been identified to modulate several aspects of sterol homeostasis including its uptake, storage and intracellular transport (Lin, et al., 2009). Lin, et al. (2009) showed in an experiment that Ste20 and Cla4 negatively regulate sterol biosynthesis through enzyme inhibition where levels of sterol, ergosterol and total sterol, were increased in cells lacking Ste20 and Cla4 as a single deletion. When either of Ste20 or Cla4 was over expressed in a multicopy plasmid, cells carrying overexpressed Ste20 showed a reduced level of sterols while those of the Cla4 were unchanged suggesting the Ste20 negatively modulates sterol biosynthesis.

In the storage of sterols via steryl esters, Cla4 was identified to negatively modulate the formation of steryl esters as increased levels of steryl esters were measured in cells lacking Cla4 while an unchanged level compared to the wild type was observed in cells lacking Ste20 (Lin, et al., 2009). When activity levels of Are2, the dominant and active form of the acyl-CoA:sterol acyltransferase under aerobic condition, were measured in cells lacking Cla4, Are2 activity levels were unchanged but when Cla4 was overexpressed in a multicopy plasmid, Are2 activity levels were reduced. This suggests the negative effect of Cla4 on sterol biosynthesis and storage. Deletion and overexpression of Ste20 has no effect on levels

of steryl esters and its synthase activity albeit, Ste20 phosphorylates Are2 (Lin, et al., 2009; Ptacek, et al., 2005).

Finally, Ste20 and Cla4 have been shown to regulate not just the synthesis and storage of sterols but its uptake as well. Ste20 and Cla4 regulate sterol import via Sut1, a transcriptional regulator that modulate uptake of sterol (Lin, et al., 2009).

1.3.2.5 Role of PAKs in vacoular inheritance

In *Saccharomyces cerevisiae*, vacoules play very important role in the cell. It serves as the site of macromolecular degradation also serving as the primary site of storage for amino acids and phosphates (Klionsky, et al., 1990). In the budding yeast, vacoules are a low copy number organelles under normal physiological condition and they require carefully co-ordinated transport to ensure their inheritance by the daughter cells (Seeley, et al., 2002).

During cell division, it is important for organelles and structures in the mother cell to be replicated in the daughter cell which applies to vacoules. At the onset of bud emergence, an important event in the cell division cycle in yeast, vacoule transport is possible only when attached to a myosin V motor, Myo2 (Catlett & Weisman, 2000). This attachment is made possible by a vacoule-specific Myo2 receptor, Vac17, which then binds to Myo2 and another vacoule membrane protein, Vac8, completing the signal for vacoule transportation to the bud. This whole complex forms a part of what is called the segregation structure. The complete transport of vacoule to daughter cell is regulated by the spatial degradation of Vac17 in a bud specific manner and this is regulated by proteins and factors that are specifically localized or activated in the bud (Weisman, 2003).

The budding yeast PAKs, Ste20 and Cla4, have been reported to be localized and activated in the bud (Bartholomew & Hardy, 2009). Cla4 and Ste20 were both shown to play crucial roles in the inheritance of vacoule by the daughter cell (Bartholomew & Hardy, 2009). Cla4 was shown to mediate the resolution of segregation structure which ensures the transport of vacoule in the mother cell to the newly developing daughter cell. Cla4 was reported to mediate this resolution through its localization to the segregation structure in the bud. Due to the overlapping function between Ste20 and Cla4, this role of Cla4 was carried out in a *cla4-75 Δste20* mutant and when the resolution of the segregation structure was investigated in this strain, segregation structure was observed to be intact as opposed the wild type strain where segregation structure was observed to be resolved. Overexpression of Cla4 and Ste20 were shown to negative regulate the degradation of Vac17, a key protein in the transport of vacoule to daughter cells (Bartholomew & Hardy, 2009; Yau, et al., 2017). Cla4 and Ste20, when overexpressed, leads to a premature destruction of Vac17 which prevents the inheritance of vacoule by the daughter cell.

1.4 Metabolism of biomolecules

In this study, Ste20 and Cla4 will be investigated on whether they regulate glucose metabolism. Recently, attention has been placed on investigating the role of Ste20 and Cla4 in the area of cellular metabolism as some reports have indicated their role in sterol metabolism and homeostasis (Lin, et al., 2009; Tiedje, et al., 2007). In an unpublished report, split ubiquitin screen identified 64 proteins that bind to Ste20, among them were 4 glycolytic enzymes. Also in humans, Pak1, the closest homologue to Ste20 in yeast, have been shown to interact and inhibit phosphoglycerate mutase (PGAM) and as such inhibits glycolysis (Shalom-Barak & Knaus, 2002). In all living cells, the utilization of biomolecules for optimum physiological function of the cell is essential. Cells have evolved numerous ways in sourcing and regulating the amount of required biomolecule depending on both internal and external conditions of such cell. Over the years, there has been a substantial study in the way cells break down (catabolise) and build up (anabolise) biomolecules to meet up with cellular demands. The study of the anabolism and catabolism of biomolecules and the pathways involved is collectively known as metabolism.

Much of our understanding of the concept of metabolism, including pathways, regulation and dysregulation, in eukaryotes have been through studies performed in yeast (Shahin, et al., 2015). Over the years, yeast have been employed in the study of both molecular and cell biology. One of the central theme of these studies have been the metabolism of biomolecules essential to cellular development such as Lipid metabolism (Aline & Howard, 2012), protein metabolism (Petranovic, et al., 2010), carbon or energy metabolism (Locasale, 2018). Here, glucose metabolism will be addressed including its regulation, genes involved and pathophysiology of the abnormal regulation of glucose.

1.4.1 Glucose metabolism

Every cell requires energy to carry out important physiological and biochemical processes that is characteristic of living cells such as cell proliferation. Many known biomolecues, such as lipids, amino acids, can be used as a source of energy by different cells at different times. One biomolecule, glucose, serves as a primary source of energy currency in most, if not, all known living organism ranging from unicellular microorganisms, such as bacteria, to the more complex higher eukaryotes, such as mammals (Towle, 2005). Glucose is not only important because of its energy generating properties, it serves as a metabolic substrate for the biosynthesis of other biomolecules, such as lipids and amino acids and a signalling molecule that regulates physiological and pathological processes (Busti, et al., 2010 and Santangelo, 2006). Due to its physiological importance, cells have evolved different mechanisms in sensing and regulating the availability and scarcity of glucose (Towle, 2005) as well as transducing this information internally resulting in the regulation of glucose uptake, gene expression, cell division, activation and/or deactivation of downstream signalling proteins etc (Takeda, et al., 2015).

Much of our knowledge on the metabolism of glucose has been attributed to studies carried out on *S. cerevisiae* (Conrad, 2014). *S. cerevisiae* uses glucose, as its preferred source of carbon in the generation of energy (ATP) and to carry out other important physiological processes related to carbon (B. Turcotte, 2010). In *S. cerevisiae*, as well as in higher eukaryotes such as humans, regulation of glucose metabolism is important to the optimum function of cellular activities as abnormality in regulation has been reported in several pathological processes such as diabetes mellitus (Aronoff, et al., 2004.) and tumor metastasis (Shaw, 2006). Regulation of glucose metabolism begins with cells ability to sense

the availability of glucose from their environment. Cells respond to excess or scarcity of glucose through several processes including gene expression and protein regulation (Schüller, 2003).

In *S. cerevisiae*, as well as humans, metabolism of glucose for the generation of energy and metabolic substrate for the biosynthesis of cellular constituents is mainly via glycolysis. However, other known metabolic route of glucose exist including gluconeogenesis, a directly opposing route to glycolysis; that leads to synthesis of glucose from non-carbohydrate moiety (Tripodi, et al., 2015), pentose phosphate pathway (PPP or P3) and glycerol synthesis. Metabolism of glucose and the pathway activated depends on several factors including the availability of glucose from the cell surrounding, metabolic need of the cell etc. In humans, cancer cells have evolved ways of reprogramming the utilization of glucose by upregulating the pentose phosphate pathway via glycolysis to satisfy their molecular needs for survival (Jiang, et al., 2014). *S. cerevisiae* utilize glucose in several ways, for instance when exposed to hyperosmotic condition, they reprogramme the utilization of glucose towards the increased synthesis of glycerol (Nevoigt & Stahl, 1997).

Here, metabolism of glucose and its regulation will be discussed under the following; glycolysis, gluconeogenesis, pentose phosphate pathway and glycerol synthesis.

1.4.2 Glycolysis

Glycolysis and gluconeogenesis are two centrally important metabolic routes of glucose in living cells. Glycolysis on one hand is the break down of glucose into simpler metabolites such as pyruvate while gluconeogenesis on the otherhand is the synthesis of glucose from non carbohydrate moieties (Fig. 1.8). Both are somewhat similar but different pathways in glucose metabolism as they share a number of enzymes with the exception of a few that are specific to each one.

Glycolysis, a ten step enzyme-catalysed pathway, is an evolutionary conserved metabolic pathway of glucose known to exist from the most primitive organisms such as budding yeast to the most complex multicellular organisms such as humans (Locasale, 2018). It is one of the oldest and most studied biochemical pathway. Glycolysis in all cells starts with the availability and uptake of glucose in the cell. Glucose, a six carbon molecule, is metabolized to pyruvate with the generation of energy in the form of adenosine triphosphate (ATP) and the reducing equivalent NADH along with other glycolytic intermediate molecules that serve as precursors for biosynthesis of other cellular constituents such as nucleic acids, lipids and amino acids (Locasale, 2018). In humans, the major source of glucose for glycolysis is from dietary carbohydrates and cellular glycogen. The main purpose of glycolysis however is the generation of energy. As mentioned above, glycolysis is a coordinated biochemical process that occurs in a ten sequential reaction in the cytosol (Fadaka, et al., 2017). Each of these reactions are enzyme-catalysed with seven of the reaction steps being reversible while three are irreversible (Fig. 1.8). Glycolysis is divided into two phases, owing to energy commitment and generation, the preparatory or investment and the payoff phase.

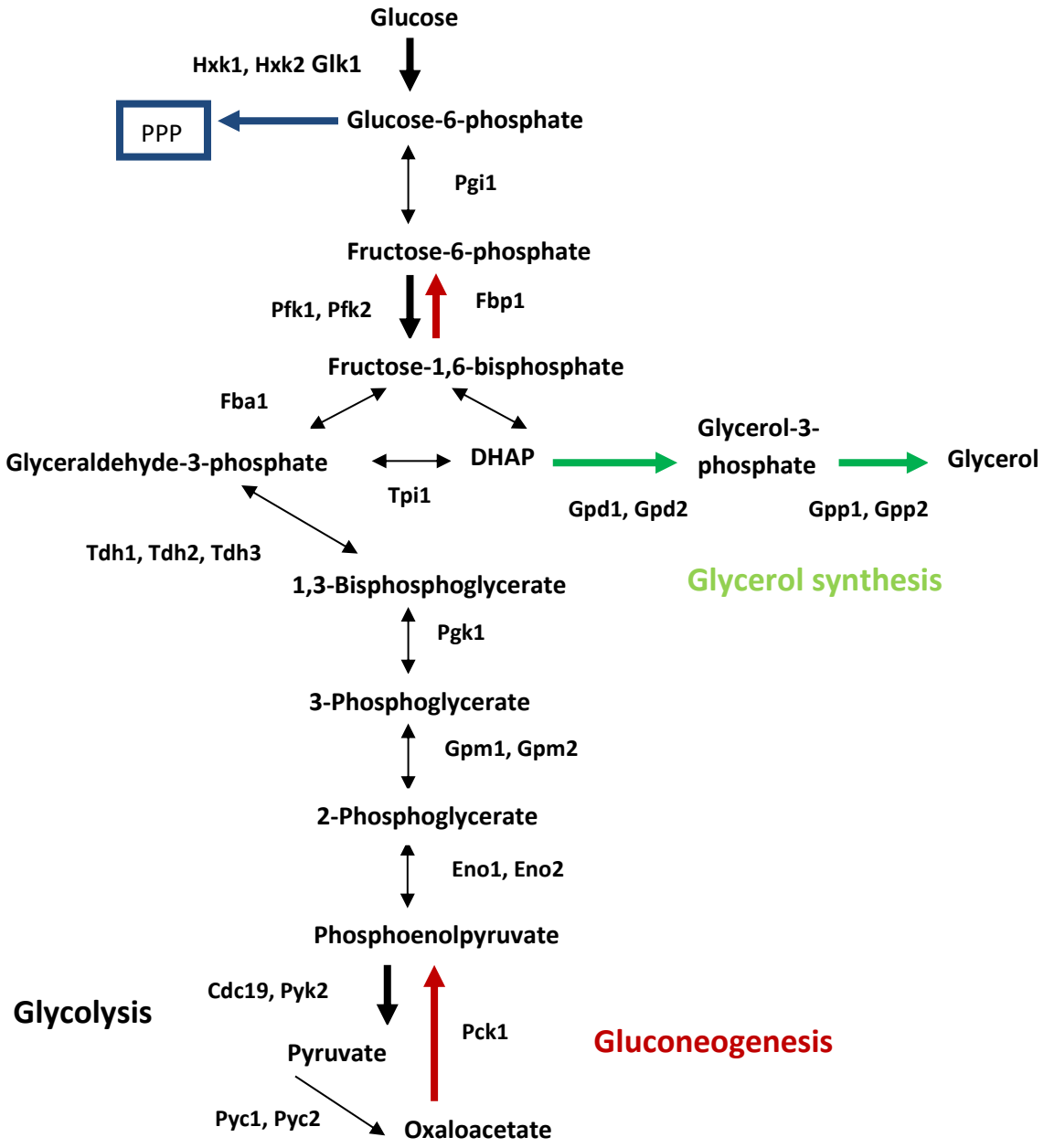


Fig 1.8. Simplified representation of glucose metabolism and the enzymes involved in budding yeast. Black code represents glycolysis, with the bold arrows indicating irreversible steps, red code shows gluconeogenesis with the bold arrows indicating those steps peculiar to gluconeogenesis. The green arrows indicate the pathway leading to glycerol synthesis while the blue arrow shows the pentose phosphate pathway (PPP pathway not shown in details here).

The investment phase of glycolysis, constituting the first five steps, is characterised by the consumption of energy in the form of ATP to commit glucose to the glycolytic pathway while the payoff phase sees the compensation of energy used in the investment phase by

producing more energy (ATP) than being used up along with the reducing equivalent NADH. Overall, glycolysis is defined by the consumption of one molecule of glucose, 2 ADP, $2NAD^+$, 2P*i* and the generation of 2 pyruvate, 2ATP, 2 NADH,

stoichiometric representation or net reaction;

$$\text{Glucose} + 2NAD^+ + 2ADP + 2Pi \longrightarrow 2\ \text{Pyruvate} + 2\ \text{NADH} + 2H^+ + 2ATP + 2H_2O.$$

The fate of pyruvate formed is determined or depends on by the presence or absence of oxygen. In the presence of oxygen i.e aerobic conditions, pyruvate is converted to two molecules of acetyl CoA which then enters the tricarboxylic acid (TCA) cycle or citric acid cycle yielding products that eventually enter the electron transport system, where the bulk of energy in glucose is harvested, approximately 32-38 molecules of ATP per glucose molecule. In the absense of oxygen i.e anaerobic condition, pyruvate is fermented to lactic acid or ethanol.

The rate of glycolysis in cells depends on some factors including cellular needs and nutrient availability. As an example, cancer cells are known to be in high demand of energy and cellular constituents such as nucleic acids to support their uncontrolled proliferation. Rather than oxidizing glucose completely via the TCA cycle and the electron transport chain, cancer cells rather increase the rate of glycolysis, fermenting glucose to lactate to produce the required energy. They do this regardless of whether there is sufficient oxygen or not, a phenomenon referred to as the Warburg effect.

This phenomenon is rather not peculiar to cancer cells but to rapid proliferating cells (Brand, 1997). *S. cerevisiae* utilizes glucose through fermentation, the most preferred lifestyle of the

organism, by increasing the rate of glycolysis to produce energy and ethanol even in the presence of oxygen, a phenomenon known as the Crabtree effect (Carlson, 1999).

1.4.2.1 Enzymatic activities and regulation of glycolysis and gluconeogenesis

As mentioned above, glycolysis is a ten step enzyme-catalysed reaction. Each enzyme carefully regulates this pathway (Fig. 1.8) and are discussed below;

- ***Hexokinase***

This is the first enzyme of the glycolytic pathway and catalyse the first irreversible reaction, which is the phosphorylation of glucose to glucose-6-phosphate. It is an energy-consuming reaction in the cell as it uses ATP in the phosphorylation of glucose. In budding yeast, three isoforms of this glucose phosphorylating enzyme exist, hexokinase I (Hxk1), hexokinase II (Hxk2) and glucokinase, Glk1, have been reported (Jasper, et al., 2001) while in mammalian tissues, four isoforms have been characterised, Hk1, Hk2, Hk3 and Hk4 (glucokinase) (Wilson, 2003). Each of these enzymes has different affinities for glucose and ATP as well as other hexose sugars. These three enzymes play a critical role in the expression and activity of each other determining which enzyme is most active under specific conditions (Voit, 2003). When yeast cells are grown in medium rich in glucose, studies have reported the role of Hxk1 in the upregulation of Hxk2 expression. Expressed Hxk2 then moves into the nucleus to repress the expression of both Hxk1 and Glk1 and further upregulates its own expression, hence establishing the role of Hxk2 in glucose repression (Rodriguez, et al., 2001). On the otherhand, when the levels of glucose are low or when yeast cells are grown on non-fermentable carbon sources, levels of Hxk1 expression become significantly reduced. However, studies have shown that overexpression of Hxk1 can replace the glucose

repression function of Hxk2 in an Hxk2 deleted cell but not Glk1 and also both Hxk1 and Hxk2 can supply the yeast cell adequate level of sugar phosphates (Rose, et al., 1991). Unlike hexokinases in other organisms, those of budding yeast , are not inhibited by the product of their reaction i.e glucose-6-phosphate.

- **Phosphoglucose isomerase**

This is the second enzyme of the glycolytic pathway, encoded by the *PGI1* gene, that produces Fructose-6-phosphate and also serves as the last enzyme of the gluconeogenetic pathway. It catalyses the isomerization of glucose-6-phosphate to fructose-6-phosphate, a reversible reaction. Studies in S. cerevisiae, have shown that cells with Pgi1 defect could not grow on glucose as it is leads to an accumulation of glucose-6-phosphate hence switching the flux via the pentose phosphate pathway, which accounts for about 8% of total growth on glucose (Dickson, et al., 1995).

- **Phosphofructokinase**

Phosphofructokinase, Pfk, catalyses the second rate-limiting and irreversible reaction of the glycolytic pathway that catalyses the ATP-dependent phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, which further commits glucose to the glycolytic pathway and provides the channel for glycerol formation. Pfk1 and Pfk2 are the two isozymes found in *S. cerevisiae* (Tripodi, et al., 2015). Pfk is an allosteric enzyme inhibited by ATP, which indicates a high supply of energy by glucose but is allosterically activated by fructose-2,6-bisphosphate, a metabolite with a high overriding power over ATP (Aleksandra, et al., 2015). In resting cells overproduction of Pfk has been shown to increase the glycolytic

flux than when compared to growing cells, as growing cells already have a well produced level of ATP required for growth (Hans, et al., 2000).

- **Fructose bisphosphate aldolase**

Fructose bisphosphate aldolase, Fba, catalyse the all important reversible cleaveage of fructose -1,6- bisphosphate to the three carbon molecules, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. This enzyme is found in all animals, plants and micro organisms such as yeast and bacteria. Two classes of aldolase have been reported, class I and class II aldolase (Roumelioti, et al., 2010). Class I aldolases are found in higher organisms while the class II are common within microorganisms such as yeast. Budding yeast aldolase is a dimeric metalloenzyme that requires the presence of a divalent metal, usually Zn^{2+}, for their activity (Berry & Marshall, 1993). Studies have shown that Fba is found in larger concentrations than required for their glycolytic function which suggests they may be required for other cellular activities (Benziane, et al., 2007).

- **Triosephosphate isomerase**

Triosephosphate isomerase, Tpi, is an enzyme that catalyse the reversible isomerisation reaction of dihydroxyacetone phosphate, DHAP, to glyceraldehyde-3-phosphate, G3P or GAP. It is an essential enzyme of glycolytic pathway. Studies where Tpi activity were reduced show an intracellular accumulation of one of its two substrate, DHAP and a drainage of the G3P hence resulting in the inhibition of glycolysis, increased activation of the pentose phosphate pathway, glycerol metabolism and lipid metabolism (Orosz, et al., 2009). Increased accumulation of DHAP due to Tpi inactivity, results in some cellular implications such as decreased production of ATP and subsequent trapping of inorganic phosphate

(Ciriacy & Breitenbach, 1979). Also, DHAP, as well as G3P, is known as a potential producer of the toxic reactive aldehyde, methylglyoxal (MG) and possess a reactive carbonyl group that can easily glycate proteins (Kalapos, 2008). In humans, the deficiency of Tpi has been reported in reduction of lifespan as accumulation of MG glycate important proteins such as those involved in cell signalling (Yao, 2006), inactivation of mitochondria (Wang, 2009) and stimulation of reactive oxygen specie (ROS) formation (Desai, 2008).

- **Triosephosphate dehydrogenase**

This enzyme catalyse the oxidative conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. It is also known as glyceraldehyde-3-phosphate dehydrogenase, GAPDH, and most of the functional studies of this enzyme have been carried out on yeast. It is encoded for by three unlinked genes in the yeast genome, *TDH1*, *TDH2* and *TDH3* (Holland, 1980). Reports have shown it is one of the most ample soluble enzymes in the cytosol (Scopes R.K., 1982). Disruption of each of the *TDH* gene such as individual deletion of each gene, double deletion of *TDH1/TDH2* and *TDH1/TDH3* shows cells are capable of growing on a glucose rich medium as a source of carbon, hence they are viable (McAlister, 1985). However, a *tdh2/tdh3* mutant shows no cell viability, indicating a functional redundancy of Tdh2 and Tdh3 while Tdh1 is suggested to be involved in cellular processes other than energy metabolism (McAlister, 1985).

- **Phosphoglycerate kinase**

Phosphoglycerate kinase, Pgk, is an important enzyme in glycolysis that catalyses the reversible reaction involving the transfer of a phosphate group from 1,3–bisphosphoglycerate to ADP, producing ATP and 3-phosphpoglycerate. This reaction step is

highly exergonic and a vital driving force for glycolysis. Recent studies have shown that this enzyme performs other cellular functions beyond glycolysis (Scatena, et al., 2008). One of the two known Pgk isoforms in human, Pgk1 has been reported to be associated with multi-drug resistance phenotype in different cancers (Wang , et al., 2010).

- **Phosphoglycerate mutase**

Phosphoglycerate mutase, PGAM, catalyse the reversible transfer of the phosphate group between the C-2 and C-3 on glycerate. In eukaryotes such as yeast and human, the isoform of PGAM present is dependent on 2,3- bisphosphpoglycerate as phosphate donor (Jedrzejas, 2000). In human, PGAM deficiency has been implicated in a rare autosomal recessive metabolic myopathy, i.e associated with the skeletal muscle, a pathological condition characterised by exercise induced cramps and myoglobinuria (Di Mauro, et al., 1981). PGAM also has been shown to have a direct link in the modulation of responses and chemoattractant signalling in enhancing human defence agsainst pathogens (Shalom-Barak & Knaus, 2002).

- **Enolase**

Enolase, a metalloenzyme, is responsible for the catalytic conversion of 2-phosphoglycerate to phosphoenol pyruvate. Three isoenzymes of enolase have been reported, α-enolase (*ENO1*) well expressed and highly conserved (Pancholi , 2001) , β-enolase (*ENO3*) exists mainly in muscle tissues, γ-enolase (*ENO2*) is associated with the neuroendocrine and nervous tissues (Ji, et al., 2016). Its biological role has been found to exceed its glycolytic function but have also been indicated in cancer, fungal infections and autoimmune diseases (Ji, et al., 2016). Studies where the *ENO1* gene was knocked out in *C. albicans,* showed

reduced cell growth, virulence and drug sensitivity of cells indicating the relevance of this enzyme in other non-glycolytic biological functions (Lo, et al., 2005).

- **Pyruvate kinase**

The last irreversible reaction step in glycoslysis is catalysed by pyruvate kinase. Pyruvate kinase is responsible for the catalytic transfer of the phosphoryl group on its substrate, phosphoenolpyruvate, to ADP yielding ATP and pyruvate – which can further be oxidized in the citric acid cycle. It is an energy-yielding enzyme serving as a checkpoint in the glycolytic process (Munoz & Ponce, 2003). In yeast, two isoforms of the enzyme have been reported, Cdc19 and Pyk2, with Cdc19 responsible for the major flux of the glycolytic step and involved in yeast fermentative growth (Boles , et al., 1997). In humans, four isoforms of this enzyme has been identified, PKM1, PKM2, PKR, PKL (Imamura & Tanaka , 1982). PKM2 is widely expressed in nearly all adult tissues while other isoforms are tissue-specific (Cardenas & Dyson , 1978). PKM2 plays a major role in cell proliferation by coordinating processes that result in the accumulation of synthetic intermediates and energy for cell division (Mazurek , et al., 2005). This proliferating role of pyruvate kinase has been reported in rapid dividing cells such as cancer cells and yeast (Christofk , et al., 2008).

So far, all these enzymes except for those that catalyse the irreversible steps in glycolysis i.e hexokinase, phosphofructokinase, pyruvate kinase, are also enzymes of gluconeogenesis. Gluconeogenesis is initiated from pyruvate, the last product of glycolysis. Pyruvate is converted to phosphoenolpyruvate via a two step reaction. The enzymes responsible for this important catalysis will be discussed below.

- **Pyruvate carboxylase**

Pyruvate carboxylase is the enzyme responsible for the ATP dependent carboxylation of pyruvate to oxaloacetate, the first and important step in the gluconeogenetic pathway. Its physiologic relevance is not limited to gluconeogenesis as it supplies oxaloacetate for the TCA cycle and NADPH for lipogenesis (Jitrapakdee & Wallace, 1999). Two isoenzymes, ,Pyc1 and Pyc2 of pyruvate carboxylase have been reported in yeast while no tissue specific isoenzyme have been reported in mammals (Walker, et al., 1991). In yeast, defects in both isoenzymes, Pyc1 and Pyc2 have been reported to result in failure of cells to grow on a glucose minimal media while mutants of either show changes in growth phenotype (Walker, et al., 1991). Defects or deficiency of pyruvate carboxylase in humans have been implicated in certain disease conditions including congenital lactic acidosis, a condition characterised by accumulation of lactate, pyruvate and alanine which results in delayed development and psychomotor retardation (Robinson, et al., 1987).

- **PEP Carboxykinase**

PEP carboxykinase, encoded by *PCK* gene, catalyse the second energy consuming step in gluconeogenesis that converts oxaloacetate to phosphoenolpyruvate. Pck1, in budding yeast, is a decarboxylating enzyme that is inactivated by glucose and activated by metabolites of the gluconeogeneic pathway and as being shown to be an important enzyme in the anaplerotic pathway in *S. cerevisiae* (Rintze, et al., 2010).

- **Frucose-1,6-bisphosphatase**

Fructose-1,6-bisphosphatase in budding yeast is encoded by the *FBP1* gene and catalyzes the third irreversible step in gluconeogenesis. It is responsible for the hydrolysis of fructose-

1,6-bisphosphate to fructose-6-phosphate. Fbp1 in the budding yeast isozyme and in the presence of glucose, its expression is repressed and rapidly degraded (Holzer, 1984). Deficiency of Fbp1, a rare autosomal disorder, prevents the production of glucose from all gluconeogenic precursor including dietary fructose and is characterised by hypoglycemia, ketosis, lactic acidosis etc (Lebigot, et al., 2015).

- **Glucose-6-Phosphatase**

The last irreversible step in gluconeogenesis is the production of glucose from the dephosphorylation of glucose-6-phosphate catalysed by glucose-6-phosphatase, Gp. Gp is not present in budding yeast but rather in liver and kidney of humans. Deficiency of Glucose-6-phosphatase is responsible for the glycogen storage disorder type I in humans characterised by hyperlipidemia, hyperlacticidemia, hepatomegaly etc (Froissart, et al., 2011).

1.4.3 Glycerol metabolism

Glycerol, a ubiquitous organic compound, is an important alcohol with a key role in carbon metabolism as widely studied in the yeast *S. cerevisiae.* Glycerol has a wide application not just in research of cell biology but also in the food, pharmaceutical, chemical and beverage industries (Scanes, et al., 1998). Higher levels of glycerol have been suggested to contribute to the viscosity and smoothness of wine and are produced on a large scale as a by product of ethanol fermentation in S. cerevisiae (Scanes, et al., 1998).

At the cellular level, the relevance of glycerol to cell survival has been established through numerous studies carried out on S. cerevisiae. When yeast cells become exposed to high salt or hypertonic medium they become stressed as a result of the efflux of water from the cytosol. Upon longer exposure without necessary cellular adjustment several cellular activities might become impaired such as inhibition of enzyme activity which could alter normal cellular activities. Several studies have shown that a rather generalised mechanism used by yeast cells and other microorganisms to overcome or counteract the efflux of water in such hypertonic environment is the accumulation and upregulation of specific compatible solutes (Brown, 1978). In yeast, one of such known and well studied solute is glycerol (Brown, 1978). Glycerol, a polyhydroxy alcohol, is the most compatible known solute in osmoregulation in *S. cerevisiae* (Bloomberg & Adler, 1992). Metabolism of glycerol has gained a significant interest over the years. Its role in both stressed and non-stressed cells has been well investigated. Glycerol has been shown to be relevant in carbon metabolism of *S. cerevisiae* in several ways (Nevoigt & Stahl, 1997). It can be used as the sole carbon source under aerobic conditions and it is also a by-product of fermentation of glucose and

other fermentable sugars to ethanol (Nevoigt & Stahl, 1997). Here, our attention is on the production of glycerol which will be discussed below.

Glycerol is synthesised in the cytosol of the yeast cell. One of the main routes of glycerol synthesis is from the glycolytic intermediate dihydroxyacetone phosphate (Scanes, et al., 1998). Dihydroxyacetone phosphate, DHAP, is converted to glycerol through a two-step enzyme-catalysed reaction (Gancedo, et al., 1968). DHAP is first converted to glycerol-3-phosphate by a cytosolic NADH-dependent enzyme, glycerol-3-phosphate dehydrogenase (Gpd) (Albertyn, et al., 1992). Glycerol-3-phosphate is then dephosphorylated by a specific phosphatase, glycerol-3-phosphatase (Gpp). This route of glycerol synthesis is believed to be the major pathway employed by the yeast cell during osmoregulation and balancing of redox potentials (Nevoigt & Stahl, 1997). A second alternative pathway to glycerol production is via the dephosphorylation of DHAP to dihydroxyacetone, DHA (Ruijter, et al., 2004) which will not be discussed in detail here.

1.4.3.1 Enzymes involved in glycerol synthesis

- **Glycerol 3-phosphate dehydrogenase**

Glycerol 3-phosphate dehydrogenase, GPD, is the first enzyme in the Gpd-Gpp pathway of glycerol synthesis and is responsible for the catalysis of the rate limiting step involving the conversion of DHAP to glycerol-3-phosphate. In *S. cerevisiae*, two isoforms of this enzyme are known, Gpd1 (Albertyn, et al., 1994) and Gpd2 (Eriksson, et al., 1995). Both isoforms have similar kinetic features but different transcriptional regulation, cellular localization and distribution (Albertyn, et al., 1994; Eriksson, et al., 1995). Studies have shown Gpd1 is actively responsible for osmoadaptation and osmoregulation as yeast strains with a single

deletion of *GPD1* showed increased sensitivity to osmotic stress (Albertyn, et al., 1994). Gpd2, has been shown to be the primary active isoform under anaerobiosis as it plays an important role in balance of redox potentials (Rep, et al., 1999). However, studies have also shown that both isoforms have a seemingly overlapping function as single deletion of either isoform did not yield a noticeable change in the level of glycerol in the cell with a study showing an upregulation of *GPD2* in a *GPD1* deleted strain (Deluna, et al., 2010).

- **Glycerol-3-phosphatase**

This enzyme is responsible for the conversion of glycerol-3-phosphate to glycerol by the removal of the phosphate group on carbon 3. Just like the GPD, in *S. cerevisiae* two isoforms of glycerol-3-phosphatease (GPP) are known, Gpp1 and Gpp2 (Norbeck, et al., 1996). Both isoforms are differentially expressed and regulated under different conditions. Gpp2 predominates and is the actively regulated isoform under osmotic stress condition while Gpp1 is upregulated under non- stress and anaerobic conditions serving primarily in redox balancing and ethanol production (Nevoigt & Stahl, 1997).

1.4.3.2 Significance of glycerol

- Glycerol and biofuels

Glycerol has found application in many fields including pharmaceuticals, beverage and food industry. Recently, due to the constant search for the replacement of the rapidly depleting fossil fuels, from which petroleum derived materials are made, with bio-based renewable sources, efforts have been made towards the study of bio-diesels and their ability as an alternative fuel option (Ayoub & Abdullah, 2012). Glycerol has been identified has an

important aspect of bio-diesel production where it exists as a co-product of the bio-diesel production process (Ayoub & Abdullah, 2012).

The physical and chemical properties as well as composition of glycerol has made it even reliable for use as a source of biofuel. Physically, glycerol is a clear, almost colourless, odourless, viscous, water-soluble, hygroscopic liquid with a high boiling point while chemically, it exists as a trihydric alcohol with a high degree of stability under most conditions. Glycerol is virtually nontoxic to human health and environment (Food and Drug Administration, 1979). The high carbon content makes it a good source of renewable energy.

Biodiesels can be produced via transesterification of triacylglycerol found in vegetable oils and animal fats with an alcohol, usually methanol, in the presence of an alkali or acid catalyst (Salamatinia, et al., 2010) yielding glycerol as by product. Glycerol produced via the biodiesel production process is crude and studies have shown the relevance of the crude glycerol in the production of value added products within the pharmaceutical and chemical industry. Some of the areas where crude glycerol has found its application includes

1. Fuel additives: crude glycerol from biodiesel production can be used in the preparation of branched oxygen containing compound by reacting with alkenes and alcohols to give such products that can serve as additives in fuels (Karinen & Krause, 2006).
2. Ethanol and methanol production: an interesting application of glycerol (including those produced from metabolic processes) is the bioconversion to ethanol and methanol. Studies have shown the cost-effective production of ethanol from glycerol using *E. coli* (Posada & Cardona, 2010). Methanol can be produced from glycerol via

hydrogenolysis of glycerol with hydrogen in the presence of a catalyst activated by a transition metal or via synthesis gas (Leoneti, et al., 2012). The produced methanol can be used again in the biodiesel production.

3. Animal feed: in recent years, use of crude glycerol as an alternative to animal feeds has gained significant attention (Leoneti, et al., 2012). In this case, vegetable oil is the raw material from which crude glycerol must be produced. In one study, the feasibility of glycerol as a replacement for corn grain feed for lactating dairy cattle was determined. The outcome of this study showed that up to about 15% dry matter of glycerol used to replace corn grain in the diet of these lactating cattle had no adverse effect on milk production or its composition (Donkin, et al., 2009).

Other applications of glycerol that can be looked up include; waste treatment, co-digestion and co-gasification, hydrogen production, green solvents for organic chemistry and cement additive (Leoneti, et al., 2012).

1.4.4 Pentose phosphate pathway

PPP or hexose monophosphate shunt or the phosphogluconate pathway is an alternative pathway to glycolysis in the metabolism of glucose. It feeds off glycolysis at the first committed reaction step, phosphorylation of glucose to glucose- 6- phosphate, (Fig 1.9). Like glycolysis, the PPP is well conserved among living organisms and according to reports, has gained significant recognition about nine decades ago when it was discovered that sufferers of the genetic disorder, haemolytic anaemia either lacked or have a reduced activity of the enzyme responsible for the catalysis of the first committed reaction of PPP, glucose 6-phosphate dehydrogenase (G6PDH) (Cordes, 1926).

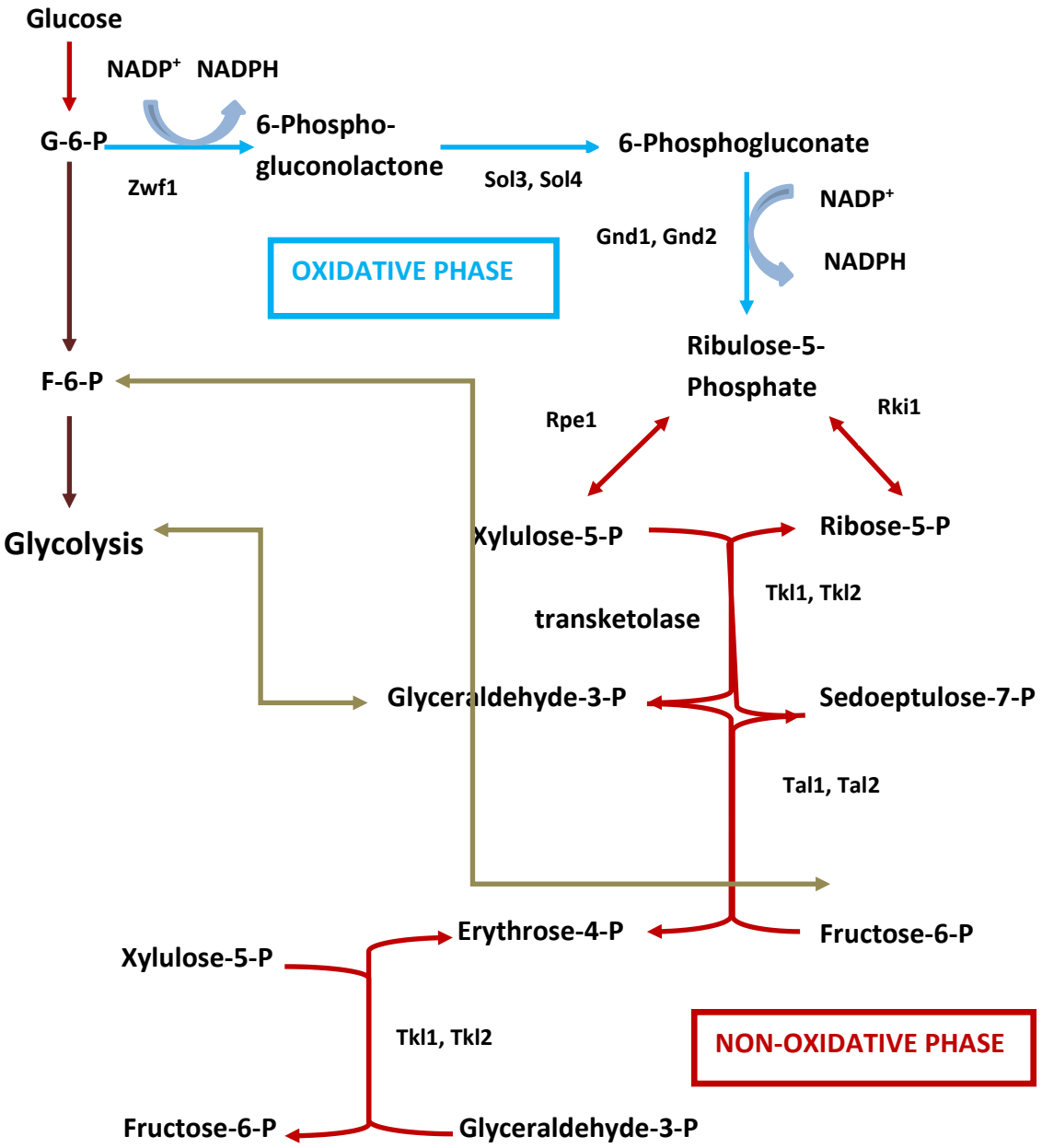


Fig 1.9. PPP and the enzymes involved in budding yeast. Blue code, shows the oxidative phase of the PPP feeding from glycolysis via glucose-6-phosphate, red code shows the nonoxidative phase of the PPP while grey code shows other points from which PPP feed from and into glycolysis.

The primary role of PPP in yeast and other eukaryotic organisms is the generation of NADPH, a cellular reducing power, required for numerous cellular reaction such as fatty acid synthesis and survival of cells under stress especially during attack by reactive oxygen species (ROS) (Patra & Hay, 2014) and phosphopentoses, that serve as precursors for synthesis of ribonucleotides including the genetic materials DNA and RNA. The PPP was one

of the earliest pathways to be discovered after glycolysis. It was first stimulated by Otto Warburg in the 1930s when he discovered the requirement of $NADP^+$ in the oxidation of glucose 6-phosphate, the first and committed step of what was later known as the PPP (Horecker, 2002). Decades later, research work carried out by Bernard Horecker and his colleagues in the 1950s that led to the full elucidation of the PPP (Horecker, 2002). This work unveiled the plethora of enzymes involved in the entire PPP. Based on this discovery and the products formed, the PPP is divided into two branches or phases; oxidative and non-oxidative phase.

In the oxidative phase of PPP (Fig 1.9), glucose 6-phosphate from glycolysis is converted to a 5-carbon phosphate moiety, ribulose-5-phosphate, through a three-irreversible enzyme catalysed reaction steps coupled with the generation of NADPH. Ribulose-5-phosphate is then isomerised to ribose-5-phosphate which feeds into and marks the initiation of the nonoxidative phase of PPP. The nonoxidative phase is composed of a series of reversible reactions, shunting more glycolytic intermediates for the purpose of generating more pentose phosphates or feeding into other biosynthetic pathways. This phase feeds off more glycolytic intermediates such as fructose-6-phosphate and glyceraldehyde-3-phosphate (Kruger & Von Schaewen, 2003). Products of this phase are very important for various cellular activities and needs. In dividing cells or rapidly dividing cells such as yeast and cancer cells, ribose-5-phosphate is required for the synthesis of DNA and RNA while erythrose-4-phosphate serves as a precursor for the synthesis of histidine and various aromatic amino acids (Cadiere , et al., 2011).

1.4.4.1 Enzymes involved in the PPP

The PPP is a highly coordinated and regulated pathway in cells due to its cellular significance. Enzymes dictate the rate at which PPP occurs and proceeds. It is therefore important to understand what they do and how they are regulated. Here, these enzymes will be discussed based on the phase of PPP they catalyse (Fig. 1.9).

1.4.4.2 Enzymes of the oxidative phase

- **Glucose 6-phosphate dehydrogenase, G6PDH**

G6PDH catalyses the rate-limiting step of the oxidative phase of PPP which converts glucose 6-phosphate to 6-phosphogluconolactone with the generation of NADPH. In *S. cerevisiae*, this enzyme is referred to as Zwf1. This step is key as it commits glucose to the PPP and as such is tightly regulated. NADPH/$NADP^+$ ratio is a strong regulator of the activity of this enzyme as high levels of NADPH negatively regulate the activity of this enzyme while $NADP^+$ is essential for its enzymatic and biochemical activity (Au, et al., 2000). It has been reported that in cells with a high demand for NADPH such as rapid dividing cells like cancer cells and yeast cells, G6PDH is high in activity and expression (Ayala, et al., 1991). Also the activity and expression of G6PDH have also been reported to be upregulated by several extracellular signals and signalling factors such as growth factors including epidermal growth factor and platelet derived growth factor as well as pro-oncogenic signalling pathways (Stanton, et al., 1991; Tian , et al., 1994).

- **6-Phosphogluconolactonase, 6PGL**

6PGL was discovered as a result of the observation that the conversion of 6-phosphogluconolactone to 6-phosphogluconate occurs at a relatively slow rate under non

enzymatic reactions which does not correlate with the rate in the cell under normal physiological conditions (Brodie & Lipmann, 1955). Its physiologic role has been reported in red blood cells where a mutation in this enzyme results in haemolytic anaemia (Beutler, et al., 1985).

- **6-phosphogluconate dehydrogenase, 6PGDH**

6-phosphogluconate produced from the second step of the oxidative phase undergoes an oxidative decarboxylation to yield a 5-carbon phosphate molecule, ribulose 5-phosphate, which signals the end of this phase and the start of the non-oxidative phase. This reaction generates the second molecule of NADPH of the oxidative phase. Together with G6PDH, the role of this enzyme is particularly important in rapid proliferating cells such as cancer cells and in the detoxification of ROS. In a particular case of cancer, 6PGDH has been reported to be critical for proliferation and tumorigenic potential (Sukhatme & Chan, 2012). Deletion of the gene encoding this enzyme or silencing of the enzyme has been reported to result in the accumulation of ROS and other metabolites upstream of its reaction point with the exception of NADPH, which remained the same. This unchanged level of NADPH could be explained for by the upregulation of G6PDH activity as the ratio of $NADP^+$/NADPH is briefly increased (Patra & Hay, 2014)**.**

1.4.4.3 Enzymes of the non-oxidative phase

- **Ribulose-5-phosphate isomerase and epimerase, RPI and RPE**

The onset of the non-oxidative phase of PPP is characterised by the conversion of ribulose 5-phosphate to ribose5-phosphate and xylulose-5-phosphate by ribulose-5-phosphate isomerase (RPI) and ribulose-5-phosphate epimerase (RPE) respectively. Ribose-5-

phosphate is a precursor molecule for nucleotide synthesis required for synthesis of DNA and RNA.

- **Transketolase (TK) and Transaldolase (TA)**

TK and TA catalyse the series of interconversion reactions that characterise the non-oxidative phase of the PPP. They both can determine the flux of metabolites in the PPP, although in a temporal manner (Patra & Hay, 2014). The activity of these enzymes is dependent on cellular needs and physiological conditions. When cells are exposed to factors that promote oxidative stress, processes that result in the generation of more NADPH are favoured as they are required for the reduction of glutathione. Under such conditions, TK converts ribose-5-phosphate and xylulose-5-phosphate to glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate while TA transfers a C3 unit from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate to form erythrose-4-phosphate and fructose-6-phosphate. A second transketolase also exist that converts xylulose 5-phosphate and erythrose-4-phosphate to form fructose-6-phosphate and glyceraldehyde-3-phosphate. Both glyceraldehyde-3-phosphate and fructose-6-phosphate are fed back into glycolysis where the latter can be converted to glucose-6-phosphate and be used up in oxidative phase of PPP to generate more NADPH while the former can downstream of the glycolytic pathway or converted to its isomeric form dihydroxyacetone phosphate for glycerol synthesis. In rapidly dividing cells such as cancer cells, the need for ribonucleotides exceed that of NADPH and as such TK and TA catalyse the reverse reaction and feed off glyceraldehyde-3-phosphate and fructose-6-phosphate from glycolysis to the non-oxidative phase of PPP to generate more ribonucleotides as ^{13}C trace experiments have shown that about 80% of ribonucleotides required by cancer cells are obtained via the non-oxidative phase of the PPP

(Boros, et al., 1997). It has been shown that cancer cells elevate the expression of TK to meet this need (Liu, et al., 2010).

1.5 Regulation of enzymes involved in glucose metabolism

Cells constantly experience changes in both their internal and external environment leading to changes in the expression of proteins as well as enzyme activity. Protein expression and enzyme activity are either increased or decreased depending on the condition a cell is exposed to and it is important for these changes in protein expression or enzyme activity to be returned to their normal levels when the conditions that have initiated them has been withdrawn or normalised. Hence, a sort of regulatory mechanism must be put in place to ensure the quick and rapid adjustment of these proteins in response to both internal and external signals as the absence or dysfunction in the regulation of such could lead to a number of cellular abnormalities including cancer and apoptosis.

Several mechanisms exist through which metabolism of biomolecules are regulated within the cell and for the sake of this work, the following will be discussed briefly; modulation of mRNA stability, allosteric regulation and post translation modifications.

1.5.1 Modulation of mRNA stability

One of the most critical mechanisms of regulation of biomolecules is the regulation of mRNA turnover. (Tripodi, et al., 2015). Using glucose metabolism as an example, when glucose is available in the cell, several yeast mRNAs are rapidly degraded (Tripodi, et al., 2015). Among those known to be degraded are those involved in gluconeogenesis such as Fbp1 and Pck1 (Mercado, et al., 1994). During this period of glucose availability, genes

encoding glycolytic enzymes and fermentation are rapidly transcribed and their mRNAs stabilized.

Certain factors contribute to the stability of mRNAs and one of such in the case of regulation of glucose metabolism is the protein kinase Snf1/AMPK. Snf1 kinase mediates a number of cellular process including regulation of phospholipid and fatty acid biosynthesis, cell cycle, invasive growth, metabolism of non-fermentable substrates (Tripodi, et al., 2015). Snf1 has been reported to mediate the regulation of mRNA stability via an Snf1-dependent phosphorylation of proteins involved in mRNA metabolism such as Ccr4, a major cytoplasmic deadenylase (Braun, et al., 2014). It has been shown that the rapid decay of the mRNAs of several glucose-repressible genes such as Fbp1 and Pck1 is because of the inhibition of the Snf1 kinase/AMPK (Young, et al., 2012).

Another important aspect of mRNA stability as a regulatory mechanism is the regulation of gene transcription. Transcriptional regulation of genes controls the abundance and level of mRNA and its translated protein product. An example of such case is seen in the adaption to osmotic stress in budding yeast via the Hog1-MAPK pathway where there is a transient upregulation of transcription of *GPD1* and *GPP2* genes to produce glycerol when grown in glucose.

1.5.2 Allosteric regulations

This mechanism of regulation of metabolism involves the non-covalent binding of a molecule, metabolite etc, on a protein causing a conformational change in the protein structure and altering its activity level in response to changes in level of a metabolite or metabolites. It is an important regulatory mechanism in glucose metabolism especially in

the regulation of glycolysis. In glycolysis, one of the key allosterically regulated steps is the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate. This reaction is catalysed by phosphofructokinase, Pfk1 and Pfk2. The enzymatic activity of phosphofructokinase is regulated by two mechanisms; (1) they are inhibited by ATP, when the cellular ATP/AMP ratio is high and are activated by AMP when this ratio is lowered. (2) they are stimulated by fructose-2,6-bisphosphate produced by 6-phosphofructo-2-kinases, Pfk26 and Pfk27 (Tripodi, et al., 2015). Another allosteric regulation in glycolysis is the feedback inhibition of hexokinase by trehalose-6-phosphate when there is an excess level of sugar phosphates (Blazquez, et al., 1993).

1.5.3 Post-translational modifications (PTMs)

PTMs as a regulatory mechanism involves the insertion of either an ion, molecule or a combination of both to an expressed protein. PTMs allow the cell to adjust to changes in both internal and external environment in a fast, dynamic, steady and reversible way by modulating protein activity (Tripodi, et al., 2015). PTMs allow for the modification of a number of enzymes at the same time which alters the nature of a given regulatory network. The following PTMs will be discussed here; phosphorylation, lysine ubiquitination and lysine acetylation.

1.5.3.1 Phosphorylation

This is one of the most widespread forms of PTMs and well-studied, especially in yeast and mammals. Proteins, such as are enzymes, can be phosphorylated in more than one position to alter their structure and activity (Sharifpoor, et al., 2011). Phosphorylation of protein is usually achieved by the action of kinases and reversed by removal of the phosphate group

by phosphatases. Phosphorylation has the capacity to altering a number of properties of the protein such as its stability, kinetics, dynamics, physico-chemical properties such as protein-protein interaction (Johnson, 2009). Phosphorylation is a vital regulatory mechanism in glucose metabolism, Pfk1, Pfk2 and Cdc19, enzymes responsible for the catalysis of two irreversible steps in glycolysis have been shown to be phosphorylation hubs, meaning they are targets of many kinases (Tripodi, et al., 2015). This observation further indicates that the reaction steps they catalyse are the most regulated in glycolysis and is essential for the cell.

1.5.3.2 Lysine modification: ubiquitination

One important way of regulating cellular processes involves the turnover rate of a protein. When a signal transduction process has been effectively acted upon by the cell, it is important for protein molecules to be inactivated or degraded to prevent them from further acting in the absence of the activating signal. Protein degradation is a common occurrence within the cell and one signal that commits a protein for degradation is by modulating the stability of such proteins. A common system within the cell that modulates the stability of proteins is the ubiquitin/proteasome pathway (Tripodi, et al., 2015), a process known as ubiquitination. Ubiquitin, a 76-amino acid residue, is a widely expressed protein in the cell that mediates the degradation of proteins by altering their stability. Ubiquitin mediated degradation begins with the binding of a ubiquitin molecule to an internal lysine of the target protein via a three-step enzymatic cascade involving E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligating enzyme, E1-E2-E3 (Swatek & Komander, 2016). The ubiquitinated protein is recognised and degraded by the 26S proteasome.

1.5.3.3 Lysine modification: acetylation

Another Important mode of cellular regulation associated with lysine modification is acetylation of lysine residues on proteins. Acetylation is the transfer of acetyl CoA to lysine residue and this mode of regulation depends heavily on the concentration of acetyl CoA – a metabolite obtainable from at least three key pathways in yeast; (1) enzymatic action of acetyl CoA synthetases, Acs1 and 2, responsible for the catalytic ligation of acetate and CoA and the hydrolysis of ATP to AMP and pyrophosphate (Tripodi, et al., 2015) (2) conversion of pyruvate to acetyl CoA by the mitochondrial dehydrogenase complex (Tripodi, et al., 2015) (3) β-oxidation of fatty acids in the peroxisomes of yeast (Hiltunen, et al., 2003). Acetylation has been best characterised in relation to events leading to transcription where several studies have shown histone acetylation as a facilitator in gene activation where transcription factors can recruit acetyl-transferase-containing cofactor to DNA complexes (Kurat, et al., 2014).

1.6 Aims

In an unpublished report, Ste20 has been shown to interact with four glycolytic enzymes. The aim of my study is to investigate the relationship of the PAKs Ste20 and Cla4, with the enzymes involved in glycolysis, gluconeogenesis, glycerol synthesis and pentose phosphate pathway. The first aim of this study is to screen for interactions between enzymes of these glucose metabolic pathway and PAKs using the split ubiquitin technique.

For the second part of this study, the aim is to find out whether Ste20 and Cla4 regulate glycerol metabolism. This will be examined under conditions in which Ste20/Cla4 play important roles including pheromone response, filamentation and hyperosmotic stress response. The expression of Gpd1, the protein that catalyses the rate-limiting step of glycerol synthesis will be investigated in a single deletion strain of each PAKs protein, Δ*ste20* and Δ*cla4* and a Δ*ste20* Δ*cla4* double mutant strain.

Finally, as kinases, Ste20 and Cla4 mediate their biochemical role by transferring phosphate group to their effectors. Whether Ste20 and Cla4 affect the phosphorylation status of Gpd1, a phosphoprotein, will be investigated. Phosphorylation status of Gpd1 will be analysed in both single deletion strain of Δ*ste20* and Δ*cla4* and *ste20 cla4* double mutant strain Also, levels of glycerol, both intracellular and extracellular, will be measured under the same conditions where the expression and phosphorylation status of Gpd1 are investigated.

Chapter 2:
Materials and methods

2.1 Materials

2.1.1 Equipments

Shaker Incubator Series Innovator® 44	New Brunswick Scientific, UK.
30^{o}C Incubator	Genlab Limited, England.
Hitachi U-1900 Spectrophotometer	Hitachi High Technologies, Tokyo Japan.
Tetrad® 2 Reltier Thermal Cycler	BIO-RAD laboratories, USA.
Gel Doc™ EZ Imager	BIO-RAD laboratories, USA.
Scout™ Pro weighing balance	OHAUS Corporation, USA.
Topmix vortex	Fisher Scientific, UK.
AccuBlock™ Digital Dry Bath	LabNet International Inc. USA.
Grant Heating Block	Grant Instruments Ltd, England.
Magnetic Stirrer	Fisher Scientific, UK.
Stuart® Roller Mixer	Bibby Scientific Ltd, UK.
Peq Power	PeqLab Biotechnologie, GmbH.
PAGE Mini-Protean® Tetra System	BIO-RAD laboratories, USA.
Microwave	Daewoo, UK.
MSE Mistral 3000i centrifuge	Mistral, England.
FRESCO 21 table top centrifuge	Heraeus, Germany.

Stuart® See-Saw Rocker	Bibby Scientific Ltd, UK.
Leica DM4000 microscope	Leica Microsystems UK.

2.1.2 Reagents and Chemicals

All restriction enzymes, DNA Taq polymerase (5000U/ml), Deep Vent polymerase (2000U/ml), Quick-Load® 1kb DNA Ladder (50µg/ml) and 6x DNA loading dye, NEB (1,2,3,4) buffers, BSA(10mg/ml), ligation buffer, T_4 DNA ligase (400,000U/ml), 10× standard Taq (Mg free) buffer, 25mM $MgCl_2$ buffer, Colour protein standard broad range were purchased from New England BioLabs. Primers were designed by me and were purchased from Eurofins Genomics. SYBR Safe DNA Gel stain (400µl, 10,000X in DMSO) and Salmon Sperm DNA Invitrogen, Acrylamide 40% solution (Acrylamide: Bis-Acrylamide 37:5:1), Agar, Tween-20, Tween-80, Tris base, NaCl, SDS (Sodium dodecyl sulphate), TEMED, Ampicillin, Mercaptoethanol and agarose were purchased from Fisher Scientific. ECL solutions A and B (Lumigen™ TMA-6) used for developing and visual imaging of blot were purchased from GE Healthcare Ltd, UK. Zymolyase 20T (from *A. luteus*) was purchased from Seikagaku Biobusiness, Tokyo Japan. Bacto™ Peptone, Yeast Nitrogen Base without Amino Acid, Bacto™ Yeast Extract were all purchased from Becton Dickson, USA. Phos-tag™ AAL-107, was purchased from Wako chemicals, Germany. α-Cdc11 (Rabbit polyclonal IgG; 200µg/ml), α-factor from 2B Scientific, α-HA (12CA5 mouse monoclonal IgG; 1mg/ml) from Roche Diagnostics, α-mouse horseradish peroxidase (1:5000, Pierce Biotechnology) and goat α-rabbit peroxidase (Jackson ImmunoResearch Laboratories). 1NM-PP1, was purchased from Cayman chemical, USA. cycloheximide (100µg/ml, Sigma-Aldrich).

2.1.3 Kits

EnzyChrom™ Glycerol Assay Kit (EGLY-200)	Bioassay Systems, USA.
Nucleospin gel and PCR clean up kit	Macherey-Nagel, Germany.
Nucleospin Plasmid kit	Macherey-Nagel, Germany.

2.2 Growth conditions, media and solution composition

2.2.1 Yeast and *E. coli* growth conditions

E. coli cells were grown in LB medium with ampicillin (100µg/ml) at 37^{o}C at 200rpm in a shaker incubator.

Yeast cells were grown in YPD or selective media according to the type of strain of budding yeast. In this study cells were grown in 10ml of media at 30^{o}C at 200rpm in a shaker incubator overnight. Cells were diluted and grown to the desired OD for another 2-3hours. Cells were harvested and transformed according to the protocol of the experiment.

For *E. coli* growth on agar plates with LB and ampicillin, cells were incubated at 37^{o}C overnight while yeast cells were grown in agar plates with the required nutrient in 30^{o}C incubator for 24 to 48 hours.

2.2.2 Media and buffers

- **Yeast media and plates**

Synthetic complete (SC) liquid media (1L): Yeast nitrogen base w/o amino acids 0.67%, Drop-out mix 0.2% Glucose 2%.

Drop out mix: 10× SC-His-Trp-Leu-Ura; Adenine 0.5%, Arginine 2%, Alanine 2%, Asparagine 2%, Aspartic acid 2%, Cysteine 2%, Glutamine 2%, Glutamic acid 2%, Glycine 2%, Inositol 2%, Isoleucine 2%, Lysine 2%, methionine 2%, para-Aminobenzoic acid 0.2%, Phenylalanine 2%, Proline 2%, Serine 2%, Threonine 2%, Tyrosine 2%, Valine 2%.

To make the SC-X: Take 18.3g of 10× SC-His-Trp-Leu-Ura add the appropriate amount of His, Leu, Trp or Ura; Histidine 0.1%, Leucine 0.2%, Uracil 0.1%, Tryptophan 0.1%. for example, for SC-His, add all components except histidine.

SC-plates (1L): Yeast nitrogen base w/o amino acids 0.67%, Drop-out mix 0.2%, Glucose 2%. Add water to make up to 400ml and autoclave. Agar 1.5%, add water to make up to 600ml and autoclave. Allow mixture to cool, add SC- liquid media to the agar and stir well using a stirring bar.

SD: Yeast nitrogen base w/o amino acids 0.67%, Glucose 2%, adenine 0.002%, Uracil 0.002%, tryptophan 0.002%, Histidine 0.002%, Leucine 0.01%, lysine 0.003%. To make a specific SD, for example SD-leu, add all the components except for leucine.

YPD: Yeast extract 1%, peptone 2%, glucose 2% and autoclave. **PLATE:** Agar 1.5%, autoclave.

YPD with G418 prepare as YPD plates, after cooling down, add filter sterilised G418 to a final concentration of 0.2mg/ml.

5-FOA plates for split-ubiquitin assay: Yeast nitrogen base w/o amino acids 0.67%, Glucose 2%, Adenine 0.002%, Uracil 0.005%, Tryptophan 0.002%, Lysine 0.003%, 5-FOA 0.1%, add 400ml water and filter sterilize. Agar 1.5%, add 600ml of water and autoclave.

Plates lacking uracil for split-ubiquitin assay: Yeast nitrogen base w/o amino acids 0.67%, Glucose 2%, Adenine 0.002%, Tryptophan 0.002%, Lysine 0.003%, add 400ml water. Agar 1.5%, add 600ml of water and autoclave.

- **Bacterial medium**

LB medium: Yeast extract 0.5%, Tryptone 1%, NaCl 0.5%, add 400ml of water and autoclave. Optionally ampicillin was added to a final concentration of 100µg/ml, ampicillin should only be added when solution is cooled. To make plate, agar 1.5%.

- **Buffers and Solutions**

20× PBS: NaCl 17.52%, KCl 4.48%, $Na_2HPO_4 \times 2H_2O$ 2.18%, KH_2PO_4 0.42%.

50× TAE: 24.2% Tris, 1.86% EDTA, adjust pH with acetic acid to pH 7.

TE: 10 mM Tris pH 8.0, 1 mM EDTA

Buffer 1: 0.9M sorbitol, 100mM EDTA, adjust pH to 7.5.

10× SDS Running Buffer: 3% Tris, 14.4% Glycine, 1% SDS.

Blotting Buffer: 20% Methanol in SDS running buffer

LiSorb: 100mM Lithium Acetate, 10mM Tris, 1mM EDTA, 1M sorbitol. pH was adjusted with HCl to 8.0 and filtered to sterilise.

LiPEG: 100mM Lithium Acetate, 10mM Tris, 1mM EDTA, 40% PEG 3350. pH was adjusted with HCl to 8.0 and filtered sterilise.

RF1: 100mM RbCl, 50mM $MnCl_2 \times 4H_2O$, 30mM potassium acetate, 10mM $CaCl_2 \times 2H_2O$, 15% Glycerol. pH adjusted to 5.8 with acetic acid. Solution was filter sterilized and kept at 4°C

RF2: 10mM RbCl , 10mM MOPS, 75mM $CaCl_2 \times 2H_2O$, 15% Glycerol. pH was adjusted to 6.8 with NaOH. Solution was filter sterilized and kept at 4°C.

PBST: 0.1% Tween-20 in PBS.

Blocking Buffer: 5% milk powder in PBS

Stripping Buffer: 0.5M Tris pH 6.8, 0.02g/mlSDS 3.5µl/ml mercaptoethanol is added before use.

ECL solution 1: 100mM tris, pH 8.5, 2.5mM Luminol, 0.4 mM p-coumaric acid

ECL solution 2: 100mM tris pH 8.5, 0.02% H_2O_2.

Ponceau S: 0.2% Ponceau S, 3% TCA**.**

TCA: 55% trichloroacetic (TCA)

2.3 Yeast strain, plasmids and oligonucleotides

2.3.1 Yeast strains

Table 1. Summary of yeast strains used in this study.

STRAINS	GENOTYPE	SOURCE
BY4742	MATα *leu2Δ0 ura3Δ0 his3Δ1 lys2Δ0 MET15*	EUROSCARF, Frankfurt, Germany
YAS8	MATa *ura3-52 lys2-801amber ade2-101ochre trp1Δ63 his3Δ200leu2Δ1Δsst1::HisG*	Dr. Thomas Hoefken
PPY966	MATa *his3::hisG leu2::hisG trp1::hisG ura3-52*	Dr. Thomas Hoefken
THY724	BY4742 *Δhem1::LEU2 Δste20::His3MX6*	Dr. Thomas Hoefken
THY737	BY4742 *Δhem1::LEU2 Δcla4::KanMX6*	Dr. Thomas Hoefken
RSY1707	BY4742 *Δhem1::LEU2*	Dr. Roger Schneiter, University of Fribourg, Switzerland
IJY1	BY4742 *GPD1-3HA-His3MX6.*	This study

IJY2-	BY4742 *GPP2-3HA-HIS3MX6*	This study
IJY3	BY4742 *Δste20::KanMX6 GPD1-3HA-His3MX6*	This study
IJY4	BY4742 *Δste20::KanMX6 GPP2 3HA-His3MX6*	This study
IJY5	BY4742 *Δcla4::hphNT1 GPD1-3HA-His3MX6*	This study
IJY6	BY4742 *Δcla4::hphNT1 GPP2-3HA-His3MX6*	This study
IJY7	YAS8 *GPD1-3HA-His3MX6*	This study
IJY8	YAS8 *GPP2-3HA-His3MX6*	This study
IJY21	YAS8 *GPD1-3HA-His3MX6 Δste11::KanMX6*	This study
IJY13	BY4742 *GPD1-3HA-His3MX6 carrying pRS425*	This study
IJY14	BY4742 *GPD1-3HA-His3MX6 carrying pRS425-STE20*	This study
IJY15	BY4742 *GPD1-3HA-His3MX6 carrying pRS425-CLA4*	This study
IJY19	YAS8 *Δste20::KanMX6 Δcla4::hphNT1 carrying YCpLac22-cla4-as3*	This study
IJY20	IJY19 *Gpd1-3HA-His3MX6*	This study

2.3.2 Plasmids

Plasmids used in this study are listed in table 2.

Table 2. Summary of plasmids used in this study.

NAME	DESCRIPTION	SOURCE
pADNX	2µm, LEU2, pADH1-N-ubiquitin	Dr. Thomas Hoefken
pFA6a-KanMX6	pFA6a carrying *KanMX6*	Wach et al. (1997)
pTH436	pADNX carrying *ENO1*	This study
pTH438	pADNX carrying *TDH1*	This study
pTH440	pADNX carrying *RPE1*	This study
pTH437	pADNX carrying *TPI1*	This study
pTH441	pADNX carrying *TAL1*	This study
pTH442	pADNX carrying *GPP2*	This study
pTH443	pADNX carrying *PCK1*	This study
pTH444	pADNX carrying *PFK2*	This study
pTH445	pADNX carrying *GPD1*	This study
pIJ1	pADNX carrying*GND2*	This study
pIJ2	pADNX carrying *FBP1*	This study
pIJ3	pADNX carrying *GLK1*	This study
pIJ4	pADNX carrying *SOL4*	This study
pIJ5	pADNX carrying *HXK1*	This study
pIJ6	pADNX carrying *GPP1*	This study
pRS425	2µm, *LEU2*	Christianson et al. (1992)

pYM1	pFA6A carrying *3HA-kanMX6*	Knop et al. (1999)
pYM2	pFA6a carrying *3HA-His3MX6*	Knop et al. (1999)
cla4-as3	YCplac22-Cla4-as3	Weiss et al. (2000)

2.3.3 Oligonucleotides

Oligonucleotides used in this study are shown below.

Table 3. oligonucleotides used in this study.

NAME	5'-3' SEQUENCE
GLK1-1	CCG CTC GAG ATG TCA TTC GAC GAC TTA
GLK1-2	ATA AGA ATG CGG CCG CAC ACG TAT GCG ATC TTA T
HXK1-1	CCG CTC GAG ATG GTT CAT TTA GGT CCA
HXK1-3	ATA AGA ATG CGG CCG CAT GAT GCA TGA AGA AGA C
FBP1-1	CCG CTC GAG ATG CCA ACT CTA GTA AAT
FBP1-3	ATA AGA ATG CGG CCG CAC TCT CCT GCT CTT CAC T
SOL4-1	CCG CTC GAG ATG GTG AAA TTA CAA AGG
SOL4-2	ATA AGA ATG CGG CCG CCA GGA GAA CTG CGT AGC T
GND2-1	CCG CTC GAG ATG TCA AAG GCA GTA GGT
GND2-2	ATA AGA ATG CGG CCG CGA CTA TAT GGC GTA CAG A

GPD1-S2	AAA GTG GGG GAA AGT ATG ATA TGT TAT CTT TCT CCA ATA AAT CTA ATC GAT GAA TTC GAG CTC G
GPD1- S3	ACA TGA AGA TCG TAC GCT GCA GGT CGA CAG AAC CTG CCG GAC ATG ATT GAA GAA TTA GAT CTA
GPP2-S2	GTA TCT GAG AAT TAT TAC TCA AAT ATG TTC GAT TTT AGA GGA TTA ATC GAT GAA TTC GAG CTC G
GPP2-S3	ATT TTT GAC GAC TAC TTA TAT GCT AAG GAC GAT CTG TTG AAA TGG CGT ACG CTG CAG GTC GAC
STE11-S1	AGC ACT TTA GTG CCA TAA AAA GAA TTA ATA AGT AGC CCT TTT TCA ATC GAT GAA TTC GAG CTC G
STE11-S2	GCC CTT GAA TTG CTG CAG CAT CCA TGG CTG GAT GCA CAC ATA ATT CGT ACG CTG CAG GTC GAC

S-primers are used for gene deletion and tagging. S1 and S2 are used for gene deletion while S2 and S3 are used for gene tagging. All other primers are used for cloning of the ORF to the vector, pADNX plasmid.

2.4 Methods

2.4.1 General guidelines

General experimental procedures employed in this research work centres around molecular biology techniques. All experiments were carried out in HNZW 139 laboratory of the College of Health and Life Sciences, Brunel University London.

To avoid contamination of samples and working materials such as pipettes, plastic ware, glass beakers etc, 70% industrial methylated spirit (IMS) was used to clean working areas and materials to be used prior to the commencement of each experiment. This was necessary as to prevent contamination of sensitive experimental procedures from the ever-present microorganisms in the surrounding atmosphere and the use of gloves to prevent contamination with DNA and RNA from living cells. Solutions for molecular biology techniques were mostly prepared under carefully controlled conditions and autoclaved to sterilize before use, while some materials such as glass ware were autoclaved to sterilize except for those purchased with a guarantee of sterility from manufacturer.

2.4.2 Molecular biological methods

2.4.2.1 DNA Amplification: Polymerase Chain Reaction (PCR)

The PCR technique allows for amplification of specific DNA sequence *in vitro*. Standard PCR reaction mixture consist of the following; deoxy nucleotide (dNTP) solution mix, buffers (required for specific polymerase), distilled water, forward and reverse primers according to the type of PCR, Taq DNA polymerase, vent DNA polymerase and template DNA (genomic or plasmid DNA, depending on the type of PCR). PCR takes place in steps; the first step of the reaction is the denaturation of template DNA, which causes the dissociation of the double

stranded DNA (dsDNA) to the single stranded DNA (ssDNA). The second step is the annealing of primers to the separated ssDNA as well as the polymerase, the enzyme responsible for the synthesis and elongation of DNA strands. The third step is the elongation of newly formed dsDNA by the DNA polymerase. The final step is the cooling or incubation of newly formed dsDNA.

2.4.2.2 PCR for gene cloning

Genes of interest of the required *S. Cerevisiae* enzymes were amplified using appropriate primers. Primers were amplified from yeast genomic DNA, BY4742 background. Cocktail mix of 100µl of PCR reagent using the following recipe in order of addition: 10µl of dNTP (2mM), 10µl of 10× buffer (-$MgCl_2$), 10µl of $MgCl_2$ (25mM), 67.5µl of H_2O, 1µl of Genomic DNA, 0.8µl Taq DNA polymerase, 0.4µl Vent DNA polymerase, 0.5µl of each primer. For concentration and details of reagents and chemicals see section 2.1.2.

Reaction mix was carried out in 0.2 ml micro centrifuge tubes and run in a thermo cycler (BIO-RAD) with a range of reaction conditions set for each reaction mix. Conditions set for this PCR reaction are as follows;

Denaturation of DNA at 95^{o}C for 5mins for 1cycle, followed by annealing of DNA strands. Temperature was set to 95^{o}C for 1min and dropped to 53^{o}C for 30secs giving each primer enough time to anneal to the denatured genomic DNA and allow for the Taq DNA polymerase to bind as well. After the annealing step, the extension of primer both in the forward (Watson) and reverse (Crick) direction was carried out at 72^{o}C for 2mins (elongation depends on the size of the PCR product, 1min/kb). The process was repeated for 29 times from annealing.

Primers and genomic DNA are kept at -20^{o}C, reaction reagents and PCR product were kept at 4^{o}C.

2.4.2.3 PCR for gene tagging

PCR epitope tagging of gene was done based on procedures described by Knop , et al., 1999. Primers S3 (forward) and S2 (reverse) are used for the gene tagging process. Primers were amplified in the appropriate plamsmid containing the desired tag. Reaction mix for the amplification of tagged gene are as described in section 2.4.2.2. PCR conditions for this reaction are listed below;

Step I**:** Initial denaturation of dsDNA (template DNA) at 94^{o}C for 2mins.

Step II: final denaturation of template DNA at 94^{o}C for a further 20secs.

Step III: annealing of primers and taq polymerase at 50^{o}C for 30secs .

Step IV: Taq polymerase catalysed elongation of newly formed DNA was carried out at 68^{o}C for 2mins. Reaction cycle was carried out for another 9 cycles from step II.

Step V: denaturation of newly formed dsDNA at 94^{o}C for 30secs.

Step VI: annealing of primers and Taq polymerase to ssDNA at 54^{o}C for 30secs.

Step VII: elongation of newly formed dsDNA at 68^{o}C for 2mins. Step V to step VII was repeated for another 19cycles.

Step VIII: incubation or cooling of PCR product and kept at 4^{o}C.

Template DNA, primers used and PCR products were stored at -20^{o}C while other PCR reagents used were kept at 4^{o}C.

2.4.2.4 PCR for gene deletion

PCR based deletion of gene was done according to procedures as described by Knop, et al., 1999 . Primers S1 and S2, forward and reverse binding respectively, were used. Primers used for gene deletion replaces the gene of interest at the open reading frame of the gene within the genome.. Reaction mixture for this PCR procedure are as described in section 2.4.2.2. Conditions set for the PCR are the same as for those for PCR for gene tagging (see sub-section 2.4.2.3).

2.4.3 Agarose gel electrophoresis, DNA gel extraction and purification

2.4.3.1 Agarose gel electrophoresis

Separation, visualisation and checking of DNA fragments were done using agarose gel electrophoresis. This was done in 1% agarose:TAE buffer with 5µl SYBR Safe gel stain and ran for 1hr at 100-150V. The mixture was gently heated to dissolve in a microwave and carefully stirred and transferred onto an electrophoretic tank and supplemented with SYBR safe gel stain for visualisation. Well teeth were gently placed in the gel to create equal dimension loading wells for the PCR product.

PCR product was purified after visualizing and size-checking by the gel electrophoresis. Purification was done according to Nucleospin PCR clean-up protocol (Macherey-Nagel) described in sub section 2.4.3.3 Purified DNA was stored at 4^{o}C.

Samples to be analysed by agarose gel electrophoresis were generally loaded accordingly;

2µl of 6×DNA loading dye was added to 10µl DNA sample in a 1.5ml tube and loaded into each well along with 5µl of DNA ladder.

2.4.3.2 DNA extraction from agarose gel

For extraction of DNA from agarose gel, gel was viewed briefly under a transilluminator and carefully cut out. Extraction and purification of DNA is carried out using the Nucleospin Gel Extraction and PCR clean up kit (Macherey-Nagel) described in sub section 2.4.3.3.

2.4.3.3 Purification of DNA

Purification of PCR products and gel extracts were done according to the protocol of Nucleospin PCR clean up kit (Macherey-Nagel). Purification steps are as follows:

2.4.4 Restriction enzyme digest.

Restriction enzyme digestion is an important aspect of molecular biology and gene cloning. Specific enzymes are used to cut DNA (dsDNA or ssDNA) at specific positions where they recognise specific DNA sequence, hence are restricted to positions where such sequence of DNA nucleotides is found. Reaction mixture for restriction enzyme digestion includes, specific enzymes, buffers (10× reaction buffers from NEB were used according to manufacturer's instruction), distilled water, bovine serum albumin (BSA) which helps to stabilize enzymes within the reaction mixture.

2.4.4.1 Digest of PCR products

For the digest of PCR products, purified DNA were used. The reaction mixture and procedure are as follows; 10µl of 10x NEB buffer was added to the purified PCR product (30-50µl, 38µl of distilled water, 1µl of BSA and 0.5µl of restriction enzyme). Reaction mix were incubated at 37^{o}C, preferably overnight for effective reaction to occur. Samples were

purified as described in sub section 2.4.3.3 and size visualized by agarose gel electrophoresis according to the procedures described in sub section 2.4.3.1

2.4.4.2 Digest of vectors for cloning

Digest of vectors for cloning were done according to the procedures below, 3µg of vector DNA were used. Between 10,000 to 20,000U/ml of restriction enzymes were added to a mixture containing, 1µl of BSA, 10µl of 10x reaction NEB buffer, 35µl of distilled water and 3µg of vector DNA. Reaction mixture was incubated at 37^{o}C for at least 3hrs.

Agarose gel electrophoresis was used to visualize the size of the product and DNA was cut off the gel and purified according to procedure described in sub section 2.4.3.3

2.4.4.3 Control digest of plasmid DNA

A control digest is done after the transformation of plasmid DNA in competent *E. coli* to be certain the DNA or gene of interest has successfully been transformed. For control digest, the reaction is made to a total of 20µl. reaction mixture includes, 2µl of 10x NEB buffer, 13.6µl of distilled water, 10,000 to 20,000 U/ml of restriction enzymes, 4µl of purified plasmid DNA. Reaction mixture is incubated at 37^{o}C for 2-3hrs.

Agarose gel electrophoresis was used to visualize the size of the product according to procedure described in sub section 2.4.3.1

2.4.5 Ligation

In molecular biology, ligation is the joining of DNA or gene of interest by DNA ligase into open ends (sticky and blunt ends) of vector DNA, which is used to transfer the gene of interest effectively into the transforming cell such as *E*. *coli*.

Purified DNA was ligated with the vector DNA, both of which have been cut, more often, by the same sets of restriction enzymes to obtain similar open ends. To achieve this, 5µl of purified PCR product was added to the reaction mixture as given below; 0.5µl of vector DNA, 1µl of 10× ligation Buffer, 0.5µL of T_4 DNA ligase. Reaction mixture was incubated overnight at 4^{o}C in the refrigerator.

2.4.6 Competent *E. coli*

Competent *E. coli* are *E. coli* cells that have been modified either by chemical or electroporation method to allow for the effective translocation of plasmid DNA into its cell.

A chemical method of making competent *E. coli* cells was employed in this work. *E*. *coli* cells were inoculated in 10ml LB medium and incubated at 37^{o}C in a shaking incubator overnight. 100ml of cells were diluted to an OD_{600} of 0.1 and grown to an OD_{600} between 0.4 and 0.6.

Cells were incubated on ice for 15mins and centrifuged afterwards at 5,000rpm at 4^{o}C for 5mins, supernatant was discarded. The pellet containing cells was resuspended in 20ml RF1 solution. Cells were further incubated on ice for 15mins and centrifuged at 5,000rpm at 4^{o}C for 5mins, with supernatant discarded. The pellet containing cells was resuspended in 4ml RF2 and incubated for another 15mins on ice.

100µl of cell aliquots were made into several 1.5 ml tubes and stored at -80^{o}C.

2.4.6.1 *E. coli* Transformation.

E. coli cells were transferred from -80^{o}C to ice. Ligation product was kept on ice while *E.coli* cells were carefully thawed and returned to ice. 100µl of *E. coli* cells were added to ligated DNA, gently mixed and incubated in ice for 45mins. Transforming cells were heated for 1min at 42^{o}C and incubated in ice for 1min. Transforming cells were loaded on LB with Ampicillin agar plates. *E. coli* cells were incubated overnight at 37^{o}C.

2.4.6.2 Isolation of plasmid from *E. coli*

Colonies of transformed *E. coli* were isolated from the agar plate and inoculated in 5ml LB supplemented with ampicillin. Cells were incubated overnight at 37^{o}C in a shaker incubator at 200rpm for plasmids to be harvested.

1.5ml of inoculants were loaded in labelled 1.5ml tubes and cells were centrifuged for 30secs at 11 000g, supernatant discarded. Isolation and purification of plasmid was done using Nucleospin plasmid kit and protocol.

2.4.7 Yeast methods

2.4.7.1 Yeast transformation

10ml of cells were grown in the appropriate media overnight in a shaking incubator. 50ml of cells were diluted to an O.D 0.1 in the appropriate media. Cells were grown for 2-3hrs at 30^{o}C in a shaking incubator at 200rpm.

Cells were centrifuged at 3000rpm for 2mins and supernatant discarded. 50ml of water was added and vortexed vigorously and centrifuged for another 2mins at 3,000rpm with supernatant being discarded. The pellet was resuspended in 10ml of LiSorb solution,

vortexed and centrifuged at 3000rpm for 2mins with supernatant discarded. The pellet was resuspended in 300µl LiSorb while 30µl of denatured carrier DNA was added (denaturation of carrier DNA; for this experiment, salmon sperm DNA was used as the carrier DNA and was incubated at 95^{o}C for 10mins and transferred to ice to cool).

50µl aliquot was transferred to labelled 1.5ml tube while 1µl of plasmid or 10µl of PCR product (for gene tagging or deletion) was added to aliquots. 300µl LiPEG was added to aliquots, vortexed vigorously and incubated at room temperature for 20mins. After the incubation process, 35µl dimethylsulfoxide (DMSO) was added to the sample and incubated for 15mins at 42^{o}C, centrifuged at 3,000rpm for 1min, supernatant discarded and resuspended in 75µl H_2O.

Cells were spread on selective plates using sterilised glass beads. Cells were incubated at 30^{o}C for several days.

2.4.7.2 Split-ubiquitin assay

The split-ubiquitin assay is a technique used to investigate the interaction between two proteins or proteins and DNA *in vivo*. The split-ubiquitin system is similar to the yeast 2-Hybrid system (Y2H) but rather overcomes the limitation encountered with the Y2H system which is limited to only soluble proteins. This technique employs the use of the two halves of the ubiquitin moiety (see section 3.1). Ubiquitin is a small regulatory protein of approximately 8.5kDa that has been found in nearly all tissues of eukaryotes (Goldstein, et al., 1975). Ubiquitin is split into the N-terminal and the C-terminal ubiquitin. Proteins to be investigated are fused to either halves of the ubiquitin moiety (Snider, et al., 2010). Attached to the C-terminal moiety is a reporter protein which is recognised by specific

deubiquinating proteases, Ubiquitin Specific Proteases (USP). Upon interaction between both proteins, the two halves of the ubiquitin assemble and reconstitute the state of the ubiquitin *in vivo* allowing for the USP to recognise and cleave off the reporter protein attached to the C-terminal ubiquitin (Snider, et al., 2010). For comprehensive discussion see section 3.1

In this study, Ste20, Cla4 and Ste14 was fused to the C-terminal half of ubiquitin moiety, which also has attached to it RUra3, a modified form of Ura3 ; an enzyme responsible for the *de novo* synthesis of uracil ,while amplified gene of interest was fused to the N-terminal half of ubiquitin. Plasmids fused to the N-terminal ubiquitin were transformed in strains already carrying plasmids in which *STE20*, *CLA4* and *STE14* are fused to the C-terminal ubiquitin according to the procedure of transformation as described in 2.4.7.1. After the transformation of the gene of interest in the stated strains of yeast, transformed cells were and grown overnight in 10ml of SC-medium lacking histidine and leucine in a shaking incubator at 30^{o}C at 200rpm. To ensure same density of cells were spotted, OD_{600} of cells were taken and divided by 100 and made up to 1000µl by adding water water. 4µl of diluted cells were loaded on SC medium plates lacking histidine and leucine and to select for the plasmids. For examination of protein-protein interaction, cells were spotted onto SC medium plates also lacking Uracil; growth on plates lacking uracil is used for examining interaction because RUra3 is the reporter probe or protein attached to the C-terminal ubiquitin. Cells with protein-protein interaction bring within proximity both halves of the ubiquitin allowing for the recognition and cleaving off of the reporter protein by USP and this translates to an inability of the cells to grow in the absence of uracil. To monitor this interaction, cells were spotted on plates lacking histidine and leucine and supplemented

with 1g/l 5-fluoroortic acid (5-FOA). when there is an interaction between proteins, growth of cells confirms interaction in the plates containing the 5-FOA. Ura3 converts 5-FOA to the toxic 5- fluorouracil that kill cells.

2.4.7.3 Isolation of yeast chromosomal DNA

A small amount of cells were resuspended in 400µl buffer-I containing 0.4µl β-mercaptoethanol and 0.025% zymolyase and incubated at 37^{o}C for 30mins. Suspended cells were centrifuged at 14,000rpm for 30secs and the supernatant discarded. The pellet was resuspended in 400µl TE, mixed with 90µl EDTA/SDS/Tris solution (50µl 0.5M EDTA, 20µl 2M Tris pH 8.0, 20µl 10% SDS) and incubated at 65^{o}C for 30mins and then incubated on ice to cool. 80µl of 5M potassium acetate was added to cell suspension and gently inverted several times to mix and incubated on ice for 1hr. Cells were vortexed and centrifuged at 14,000rpm for 5min at 4^{o}C. The supernatant was carefully transferred to a new tube with 1ml of 100% ethanol added to it, carefully mixed and centrifuged at 14,000rpm for 5mins, supernatant discarded. The pellet was resuspended in 1ml of 70% ethanol, centrifuged at 14,000rpm for 1min with supernatant discarded. DNA was dried at 42^{o}C and resuspended in 50µl of H_2O. See section 2.1 for details of reagents and chemicals, buffers and solutions used.

2.4.8 Protein techniques

2.4.8.1 TCA protein extraction

Yeast cells were grown in either 10ml YPD or SC- medium overnight in a shaking incubator at 30^{o}C at 200rpm. Cells were diluted to an OD_{600} of 0.5 and grown for 2-3hrs. After this incubation period, OD_{600} of cells were taken - for the purpose of protein analysis and

quantitation. Cells were harvested after the incubation period, centrifuged at 3,000rpm for 2mins before resuspending the pellet in 1ml of water (alternatively, cells scraped from a plate were resuspended in 1ml of water). 150µl of 1.85M NaOH was added to resuspended cells and incubated on ice for 10mins. After incubation, 150µl of 55% TCA was added and incubated on ice for 10mins. Samples were centrifuged at maximum speed at 4^{o}C for 20mins. Pellets were resuspended in 50µl of SDS or HU buffer and boiled for 15mins at 65^{o}C. Samples were centrifuged at maximum speed for 1min, supernatant transferred to a fresh tube and stored at -20^{o}C.

2.4.8.2 SDS PAGE

12% separation gel was used in the assemblage of the SDS PAGE, this is made up of 4.4ml of water, 2.5ml of 1.5M tris pH 8.8, 100µl of 10% SDS, 3ml of acrylamide, 75µl of 10% APS, 7.5µl of TEMED. Mixtures are added in order of listing as APS and TEMED are both polymerising agent and commence the process of polymerization once added. The separation gel was loaded to assembled glass blocks, 500µl of 0.1% SDS was loaded on top of the separation gel and allowed to polymerise.

The stacking gel was loaded on the separation gel. The stacking gel was made from 3.1ml of water, 1.25ml of 0.5M tris pH 6.8, 50µl of 10% SDS, 500µl of acrylamide, 30µl of 10% APS, 6µl of TEMED, gel well teeth was carefully placed on the gel and allowed to polymerise. Gels were transferred to PAGE tank and filled with SDS running buffer, 10µl of protein extracted samples were loaded in each well with 5µl protein ladder. The gel was run for 45mins at 200V. See section 2.1.2 for details on reagents and chemicals.

2.4.8.3 Western blot

The SDS-PAGE gel was prepared and transferred for blotting, setting the blotting materials in place which are made of blotting buffer, nitrocellulose transfer membrane, 2 blot papers, blotting tank.

1l of SDS running buffer was emptied in a clean tray, blot holder was placed in the tray with the blotting foam placed on it, the blot paper was placed on top of it which the gel is carefully placed on and then, the nitrocellulose membrane was placed firmly on the gel. The second blot paper was placed on the membrane while the blot foam was then laid on the whole stack and blot holder is tightly clipped and transferred to the blotting tank. The blot was run for 1hr at 100V, 350mA. After the transfer of protein to the membrane, membrane with transferred protein bands was washed with water and stained with ponceau S for 1min and washed with water again to remove excess of the stain after which the membrane is left to dry. This can be stored at 4^{o}C.

To identify tagged protein of interest, the membrane was blocked for 1hr with 5% milk powder dissolved in PBS to block proteins. The membrane was then incubated for 1hr with the primary antibody, α-HA (1:2000) in 2.5ml of 5% milk powder in PBS after which it is washed three times in PBS + 0.1% tween-20 for 5mins each. Blot was then incubated for with the secondary antibody, α-mouse horseradish peroxidase (1:5000) for 1hr in 5ml of 5% milk powder in PBS and washed three times in PBS + 0.1% tween-20 for 5mins each.

The blot was developed by incubating it in 500µl each of ECL1 and ECL 2 solutions or using the more sensitive ECL from GE Healthcare for 1min and analysed using the Chemi Doc®

analyser. For quantitation of protein bands and imaging, Gel Doc image lab 6.0.1 software was used. For details of reagents, chemicals and equipment see section 2.1.

2.4.8.4 Blot stripping

To check whether the same amount of protein samples has been loaded into each well for the SDS PAGE allowing for protein quantitation, housekeeping proteins are measured from the same blot on which our proteins of interest were transferred and/or routinely by staining with Ponceau S. To achieve this, blots are stripped of the primary and secondary antibody present on them. The stripping buffer is boiled in a water bath to a temperature of 50^{o}C.

Stripping buffer is transferred to the blot membrane placed in a container. Blot membrane is placed on a shaker for 10mins. This step is repeated one more time. The buffer is discarded and blot is washed three times for 10mins in PBST.

Same procedure of developing blot as described in subsection 2.4.3, except for the primary and secondary antibody used. Blot was incubated for 1hr with the primary antibody, α-Cdc11 (1:1000) in 2.5ml of 5% milk powder in PBS. Same procedure of washing was done as described in sub section 2.4.3. Blot was incubated for 1hr with secondary antibody, goat α-rabbit peroxidase (1:5000) in 5ml of milk in PBS solution. Same procedure of washing was done as with the primary antibody.

Blot was developed and analysed according to procedures described in sub section 2.4.3.

2.4.8.5 Phos-tag gel electrophoresis

Phos-tag gel electrophoresis is an adapted SDS PAGE technique used to identify phosphorylated proteins in a protein sample (Kinoshita, et al., 2006). Components of the Phos-tag SDS-PAGE is similar to those of the normal SDS-PAGE but differ slightly. Below are the components and compositions of the phos-tag SDS-PAGE:

Table 4: Composition of Phos-tag gels.

Composition	8%
Acrylamide	1ml
H_2O	2.6ml
1.5M Tris pH8.8	1.25ml
10% SDS	50µl
10mM $MnCl_2$	50µl
5mM Phos-tag	20µl
10% APS	25µl
TEMED	5µl

Separation gel: 8% composition was used in this study, gel was covered with butanol-saturated water and left to polymerize for about 1hr. After the gel has polymerized, the separation gel is rinsed with water.

Stacking gel: 500µl of acrylamide (40%; 37:5:1), 3.15ml H_2O, 1.25ml of 0.5M Tris pH 6.8, 50µl 10% SDS, 25µl 10% APS, 5µl TEMED. Well teeth were inserted in the loaded stacking gel and allowed to polymerize.

Protein samples were loaded in each well and gel run as normal SDS-PAGE. Afterwards, gel is soaked three times in 50ml of 50 µM EDTA in SDS running buffer: EDTA. The gel is gently washed in 50ml of blotting buffer. A PVDF membrane is used instead of Nitrocellulose transfer membrane. PVDF transfer membrane is briefly incubated in methanol as it is relatively hydrophobic, and this would impede the transfer of proteins. Same blot transfer procedure is followed as with the normal SDS-PAGE. See section 2.1 for further details on reagent and chemicals.

2.5 Experimental conditions

2.5.1 Optimal growth condition

Cells were grown overnight in appropriate medium at 30^{o}C, 200rpm shaking incubator. OD_{600} of cells diluted 1:10 were measured. Cells were diluted to an OD of 0.5 to a volume of 20ml each and grown for 2-3hours. After the 2-3hours incubation, 1ml of cells were taken each for TCA extraction and to measure cell density after which cells were returned to the shaking incubator. This was done for different time points and recorded.

2.5.2 Hyperosmotic stress

Cells were grown overnight in 10ml of appropriate medium at 30^{o}C, 200rpm shaking incubator. OD_{600} of cells diluted 1:10 were measured. Cells were diluted to an O.D of 0.4 to a volume of 20ml each and grown for 2-3hrs. After the 2-3hrs incubation, 4M NaCl was added to each cell culture and immediately 1ml of cells were transferred to cuvette for OD_{600} measurement and another 1ml for TCA extraction while cells were returned to the shaking incubator. This was done for different time points and recorded. TCA extraction was done for each point of cell harvest and expression of protein was confirmed by immunoblotting through SDS-PAGE.

2.5.3 Glucose response

Cells were inoculated in 10ml of appropriate medium overnight at 30^{o}C, 200rpm in a shaking incubator. Absorbance of cells were measured at an OD_{600}, diluted by a factor of 1:10 with water. Cells were grown to an OD_{600} 0.5 to a total volume of 15ml in YPD for 2-3hrs. 1:10 of 20% stock glucose solution was added to each cell making a final concentration of 2% glucose solution. Cells were thoroughly mixed and 1ml of cell was taken for absorbance at

OD_{600} and TCA protein extraction each, this taken as time point 0min. Same procedure of TCA protein extraction and taking of absorbance was done for different time points according to the experiment. Protein expression was confirmed by immunoblotting using SDS PAGE and western blot.

2.5.4 Cycloheximide chase assay

Cells were grown in 10ml of YPD overnight at 30°C, 200rpm in a shaking incubator. Cells were transferred to a fresh YPD medium and grown to an OD_{600} 0.5 followed by treatment with cycloheximide at a final concentration of 0.1mg/ml. Cells were grown for 4hrs, 1ml of samples were taken at every hour starting from time of treatment with cycloheximide and protein extraction was done as described in section 2.4.8.1. Protein expression was confirmed by immunoblotting using SDS PAGE and western blot.

2.5.5 Glycerol assay

Glycerol concentration was measured under different physiological conditions.

For intracellular glycerol assay, 10ml of cells were centrifuged at 3000rpm for 2min and supernatants discarded. Pellets were re-suspended in 1ml of water and transferred to a 1.5ml tube. Cells were incubated at 95°C for 10mins and centrifuged at maximum speed for 1min. Supernatants were transferred to a new 1.5ml tube. For the extracellular glycerol assay, 10ml of cells were centrifuged at 3000rpm for 2mins and pellet discarded while 1ml of supernatant was transferred to a 1.5ml tube. Samples were incubated at 95°C for 10mins. Glycerol levels were measured following instructions from manufacturer's protocol. The glycerol reaction mix comprises of the following; 100µl of assay buffer, 2µl of enzyme mix, 1µl of ATP and 1µl of dye reagent. 100µl of reaction mix is transferred into a tube containing

10µl of sample or standard glycerol and mixed. The reaction mix is incubated for 20min at room temperature and optical density of glycerol sample is measured at 570nm.

Table 5: Standard glycerol composition

No	STANDARD + H_2O	GLYCEROL (mM)
1	10µl + 990µl	1.0
2	6µl + 994µl	0.6
3	3µl + 997µl	0.3
4	0µl + 1000µl	0

Chapter 3:

Identification of protein-protein interactions using the split-ubiquitin system

3. Identifying protein-protein interactions using the split-ubiquitin system

3.1 Introduction

One of the key challenge or problem most researchers are faced with is the ability to identify how proteins interact with other proteins in their natural environment before they can successfully, through a series of experiments, elucidate their cellular functions as well as the regulation mechanism of such proteins with other proteins. In this case, one of the aims of this study is to identify and investigate the interactions between the PAK proteins, Ste20 and Cla4, with the proteins involved in the following major glucose metabolic processes; glycolysis, gluconeogenesis, glycerol synthesis and the pentose phosphate pathway. Many techniques are available to identify protein -protein interactions *in vivo* and are also important *in vitro* techniques for example using recombinant proteins. These techniques can be broadly classified as either a biochemical method or a genetic method (Fetchko & Stagljar, 2004). A biochemical method is a direct approach of identifying protein -protein interactions that establishes the state of protein interactions and then determines the composition of protein complexes while a genetic method is rather an indirect approach of determining protein interactions as it requires the manipulation or modification of endogenous or exogenous genetic make-up to give a specific visible output (Fetchko & Stagljar, 2004). Here, one of the known genetic approach, the split-ubiquitin technique of identifying protein interactions was used. The split-ubiquitin technique is a similar but more reliable, effective and an alternative option to the widely common yeast two-hybrid system (Y2H) (Johnsson & Varshavsky, 1994).

The split ubiquitin technique or system is a protein fragment complementation assay that relies on the conditional proteolysis of the protein, ubiquitin (Johnsson & Varshavsky, 1994).

This system is similar to the yeast two-hybrid system developed by Fields and Song in the 1980s (Fields & Song, 1989). The split ubiquitin system has been developed to overcome the limitations of the Y2H which only permits the investigation of protein interactions within the yeast nucleus which makes it difficult to study the interactions of proteins that are not expressed within the nucleus (Dirnberger, et al., 2008). The split ubiquitin system relies on the use of the ubiquitin molecule, a small conserved protein found in all eukaryotic cells (Doolittle , 1995). This protein marks target proteins within the cell for degradation by the 26S proteasome while the ubiquitin is saved and recycled back into the cytosol by ubiquitin-specific proteases (UBPs) (Glickman & Ciechanover, 2002). This system utilizes the fact that the ubiquitin can be separated to two halves, the N-terminal (Nub)and C-terminal (Cub) ubiquitin, with the Cub attached to a reporter, fig 3.1a (Johnsson & Varshavsky, 1994). In the natural environment of the cell, Nub and Cub of the ubiquitin spontaneously fuse together, fig 3.1a. For this reason, an amino acid, Ile-13 found in the native Nub (Nub*I*) is substituted for either alanine (Nub*A*) or glycine (Nub*G*) which reduces the affinity for Nub to spontaneously bind to Cub (Fetchko & Stagljar, 2004). When two test proteins, X and Y, under investigation are attached to the Nub*A*/*G* and Cub and interact in their natural state within the cell, this brings the Nub*A*/*G* and Cub within close proximity creating a quasi-native ubiquitin which allows for the ubiquitin-specific protease to cleave off the reporter probe attached to the Cub, fig 3.1c. A commonly used reporter is the R-Ura3 which allows for the yeast cell to synthesize its own uracil and grow in a medium lacking uracil (Fetchko & Stagljar, 2004). Ura3 is essential for uracil biosynthesis; the only Ura3 in cells used for the assay is the one fused to Cub; R-Ura3 is a modified form in which the first amino acid is replaced by an arginine (hence the R) which destabilises the protein; when fused to Cub, RUra3 is not degraded. When this R-Ura3 is cleaved off, becomes proteolytically degraded in

accordance with the N- end rule of protein pathway degradation causing the yeast cell to become incapable of growth in a medium lacking uracil hence serving as a positive selection. This also permits for the negative selection on a medium containing the Ura3 specific antimetabolite 5 -fluoroorotic acid (5-FOA). Ura3 converts the less toxic 5-FOA to the toxic 5- fluorouracil. Growth on 5-FOA therefore indicates protein-protein interaction. Aside from the split ubiquitin-technique, other techniques are available for detection of protein-protein interactions such as co-immunoprecipitation. This technique will be discussed briefly with a focus on the selection of the split ubiquitin technique over co-immunoprecipitation.

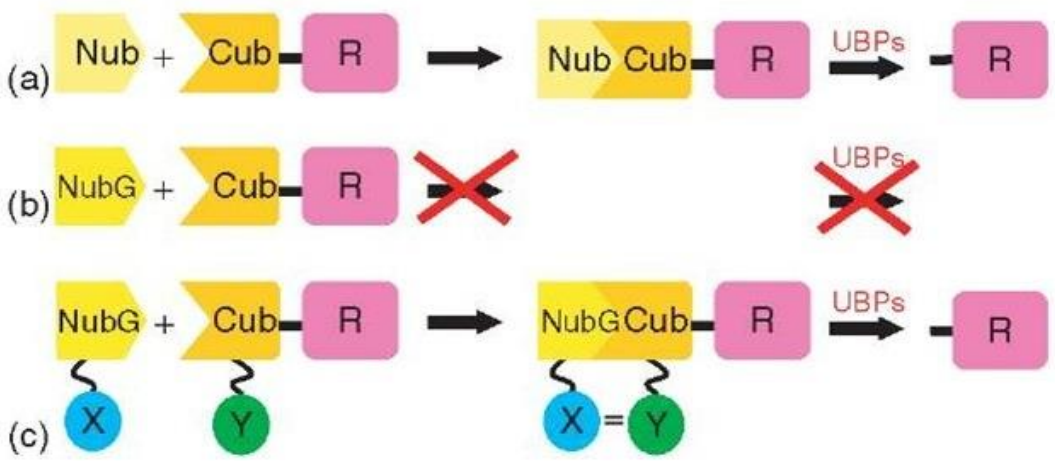


Fig 3.1. A simplified representation of the split ubiquitin system. A) shows the N- and C-terminal ubiquitin halves, Nub and Cub, in their native or natural state where they interact spontaneously, with the reporter, R, attached to the Cub. This allows for the recognition by the ubiquitin-specific proteases, UBPs. B) A mutated form of ubiquitin, where the isoleucine at position 13 of the native Nub*I* is substituted with glycine, Nub*G.* This mutation prevents the spontaneous interaction and the subsequent cleavage by the UBPs. C) The mutated form of ubiquitin carrying two interacting proteins, X and Y. This interaction causes a quasi-native interaction of the ubiquitin by bringing them within close proximity and enabling recognition by UBPs hence mimicking the reaction at A.

Co-immunoprecipitation, is a common technique in molecular biology used for the detection of protein-protein interaction including subunits within a protein complex (Miernyk & Thelen, 2008). It gives one of the most convincing evidence that two or more proteins interact *in vivo* (Monti, et al., 2005). This technique relies on the use of an antibody specific for a known protein believed to form a part of a complex with the target protein.

Once binding is established, the entire complex can be pulled out from a mixture using immobilised protein A or G. In comparison to the split-ubiquitin technique, co-immunoprecipitation is not all assuring in determination of whether an interaction is direct or indirect due to the possibility of the presence of additional proteins while split-ubiquitin measures the direct interaction between proteins void of other subunits. Also, co-immunoprecipitation is less sensitive to detect transient or weak interaction while the split-ubiquitin technique is preferred for its sensitivity in detecting very weak and/or transient interactions. This molecular advantage could be important in detecting interactions between kinases and their effector proteins. Another important advantage of the split-ubiquitin technique over co-immunoprecipitation is that it is suitable for studying interaction involving membrane proteins, which is relevant for this study because Ste20 and Cla4 can be found at the plasma membrane.

In this study, a number of enzymes of the glucose metabolic pathways are screened for interactions with Ste20 and Cla4. The reason for choosing to investigate interactions of Ste20 and Cla4 with these enzymes stems from the result of a previous split-ubiquitin screen for interactors with Ste20. This result identified 64 interactors of Ste20 (Tiedje, et al., 2007), of which only one, Bem1, has been previously characterised and known to interact. A number of these interactors have been characterised (Tiedje et al., 2007; Lin et al., 2009a; Lin et al., 2009b; Lin et al., 2012). Amongst them were also four glycolytic enzymes, Pgk1, Gpm1, Cdc19, Tdh3 (Thomas Hoefken, unpublished data). For the reason that glycolytic enzymes were over-represented in the screen, this study chose to systematically test the interaction between Ste20 and glycolytic enzymes extending this to include Cla4 and enzymes involved in gluconeogenesis, the PPP and glycerol synthesis. At the start of this

research, a few enzymes have already been tested for interactions and as such one of the aims of this study is to analyse the remaining interactions. To achieve this, genes encoding several enzymes of glucose metabolism will be cloned in a split ubiquitin vector.

3.2 Aim

- To generate and characterise protein constructs of the enzymes involved in the major glucose metabolic pathway and identify those that interact with Ste20 and Cla4 proteins using the split-ubiquitin technique.
- To establish the strength of each interaction with Ste20 and Cla4.

3.3 Results

3.3.1 Protein-protein interactions between Ste20/Cla4 and glycolytic and gluconeogenic enzymes.

Important in understanding cellular functions of Ste20 and Cla4 is the identification of the interactions of these proteins with other cellular proteins. Here, the interactions between Ste20/Cla4 and the enzymes involved in glycolysis and gluconeogenesis is identified using the split-ubiquitin system.

To analyse these interactions, the following genes, *ENO1*, *TPI1*, *TDH1*, *PFK2*, *PCK1*, *GLK1*, *FBP1* (see section 1.4.2.1 and fig 1.7), encoding the enzymes of glycolytic and gluconeogenic pathways were amplified using the PCR-based approach as described in section 2.4.2.2., and cloned into the vector, pADNX (see section 2.4.4.2 and 2.4.5), which encodes the N-terminal half of the ubiquitin. Amplified genes were digested by the same restriction enzyme used to cut the vector DNA. Digested genes were purified and ligated in the vector, pADNX. Plasmid constructs of corresponding genes were generated by transformation of ligation product in competent *E. coli* (see section 2.4.6). plasmid constructs were isolated, purified and digested for confirmation of insert of gene of interest (see section 2.4.4.3). Prior to testing for interaction, it is important to characterise these genes and their corresponding proteins. To characterise these genes, their corresponding proteins were detected by western blotting using α-HA mouse monoclonal antibody as the vector, pADNX, in which they were cloned contains an HA next to the N-ubi. Fig 3.2 shows the cloned and characterised *GLK1* and *SOL4*.

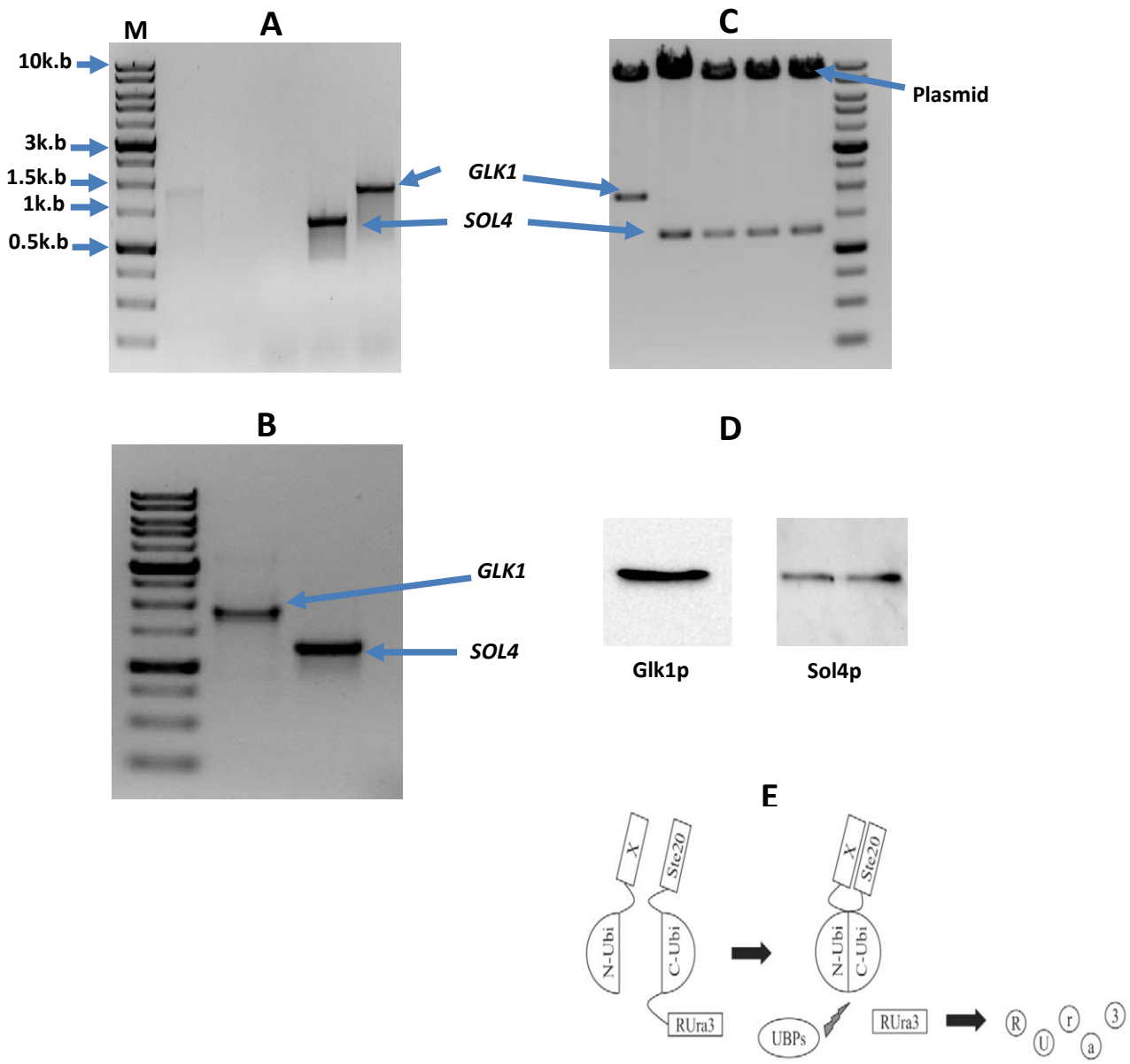


Fig 3.2. Shows an example of molecular cloning leading to the insertion to a split ubiquitin vector. A) Agarose gel result showing PCR amplification of *SOL4* and *GLK1* after which they are digested by the same restriction enzyme used for cutting the vector DNA. B) Agarose gel result showing restriction enzyme digest of *SOL4* and *GLK1* allowing for the ligation into the split ubiquitin vector, pADNX. C) Control digest of plasmid vector showing the inserts *SOL4* and *GLK1*. D) SDS PAGE western blot showing confirmation of the successful transformation of the Sol4 and Glk1 protein in yeast. E) Split ubiquitin system showing Ste20 attached to the C-ubi terminal and the reporter probe, Ura3 while our gene of interest, X, is attached to the N-ubi terminal of the ubiquitin molecule.

Constructs in which Ste20 and Cla4 are both fused to C-terminal half of the ubiquitin and tagged with a reporter protein already exist. Plasmids carrying either half of the ubiquitin fused with the Ste20/Cla4 and our genes of interest were transformed in yeast.

Transformed yeast cells were spotted on plates lacking histidine and leucine to select for the

two plasmids and to monitor growth. Selection for interactions was carried out on plates also lacking uracil. Interaction between Ste20/Cla4 and any of the test protein bringing each halves of the ubiquitin molecule within proximity allowing for the cleaving off of the reporter RUra3 protein by UBPs. This prevents the growth of transformed cells as they lack the ability to synthesize uracil. 5FOA is used as an alternative.

Seven proteins involved in glycolysis and gluconeogenesis in *Saccharomyces cerevisiae* were tested for interaction after each has been characterised and confirmed by immunoblotting. These proteins as shown in Fig 3.3 were tested for interaction with Ste20 and Cla4. Selection was carried out on plates lacking only histidine and leucine as control, where all cells grow well due to the presence of *HIS* and *LEU* genes allowing for the synthesis of the amino acids histidine and leucine required for cell growth. For positive selection of interactions, cells were spotted on plates lacking uracil along with histidine and leucine. Eno1, Tdh1, Pfk2 and Pck1, showed no growth with Ste20 which indicates an interaction with Ste20, Tpi1 and Glk1 showed slight growth with Ste20 which possibly indicating a weak or transient interaction while Fbp1 showed a very slight growth with Ste20 which possibly indicates a transient or weak interaction too and no interaction with Cla4 due to a significant growth observation. Bem1, a cell polarity protein that has been shown to interact with Ste20, was used as a positive control (no growth) while Ubc6 and Ste14 were used as negative controls (growth). With Cla4, Eno1 and Tdh1 showed weak or transient interaction while others showed no interaction.

Selection was also carried out on plates supplemented with 5FOA and lacking histidine and leucine. This was used as a negative selection because 5FOA is an antimetabolite of Ura3. Ura3 can convert the less toxic 5FOA to the toxic 5 fluorouracil which kills the cells and

prevents growth. This implies that growth in 5FOA indicates an interaction as a result of the degradation of Ura3 (no growth in plates lacking uracil) and vice versa. Here, only Eno1, Tpi1, Tdh1 and the positive control, Bem1, showed an interaction with Ste20 as growth of cells were observed for each. No cell growth was observed for others.

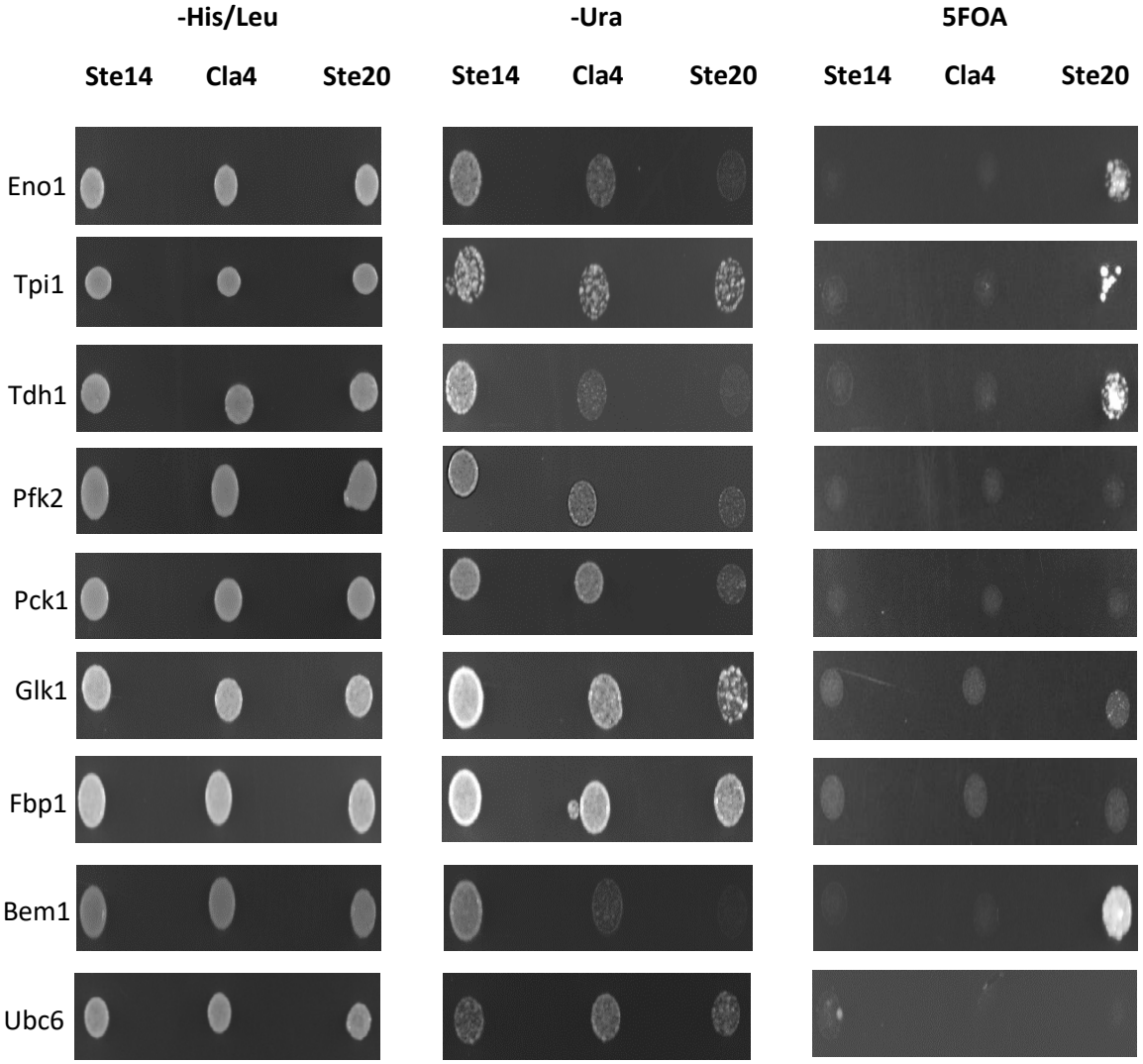


Fig 3.3 Protein-protein interaction between Ste20/Cla4 and enzymes of glucose metabolism, glycolysis and gluconeogenesis. Split-ubiquitin screening of Ste20/Cla4 and enzymes of glycolysis and gluconeogenesis. The indicated strains, Eno1, Tpi1, Tdh1, Pfk2, Pck1, Glk1, Fbp1, were spotted at a dilution of 1:10 on medium lacking histidine and leucine for growth control while selection for interaction was done on medium lacking uracil and medium supplemented with 5FOA. Cells were grown for 48hours.

Those interactions observed in 5FOA were also confirmed in the plates lacking uracil. However, for the others that appeared in the 5FOA plates as no growth, these false results could be as a result of the relatively high concentrations of 5FOA which is quite stringent

3.3.2 Protein-protein interactions between Ste20/Cla4 and PPP enzymes

Enzymes or proteins involved in PPP were also screened for interactions with Ste20 and Cla4. This is important because of the relevance of PPP as an alternative pathway in the metabolism of glucose. Four of these genes, *RPE1*, *TAL1*, *SOL4* and *GND2* (see section 1.4.4 and fig 1.8) encoding the corresponding enzymes of PPP were amplified using PCR-based approach, cloned in the vector, pADNX as described in section 3.3.1. These proteins were characterised by immunoblotting using western blot and α-HA mouse monoclonal antibody to detect the HA-tag next to the N-ubi of the cloning vector. Fig 3.2 shows the cloning and characterisation of Sol4.

Plasmids carrying either half of the ubiquitin fused with the Ste20/Cla4 and our genes of interest were transformed in yeast. Transformed yeast cells were spotted on plates lacking histidine and leucine as positive growth control. Selection for interactions was carried out on plates also lacking uracil. Also, plates supplemented with 5FOA and lacking histidine and leucine was used as an alternative.

As shown in fig 3.4, selection was carried out on plates lacking only histidine and leucine as growth control, where all cells grow well due to the presence of *HIS* and *LEU* genes allowing for the synthesis of the amino acids, histidine and leucine, required for cell growth. Selection on plates lacking uracil showed that Rpe1, Tal1, Sol4 and Gnd2 interact with Ste20 as no growth of cells were observed. Screened interaction with Cla4 showed Rpe1 and Tal1 interact with Cla4 as no significant growth was observed while Sol4 and Gnd2 showed slight growth with Cla4 indicating a weak or transient interaction. Bem1 was used as a positive control while Ubc6 and Ste14 were used as negative control.

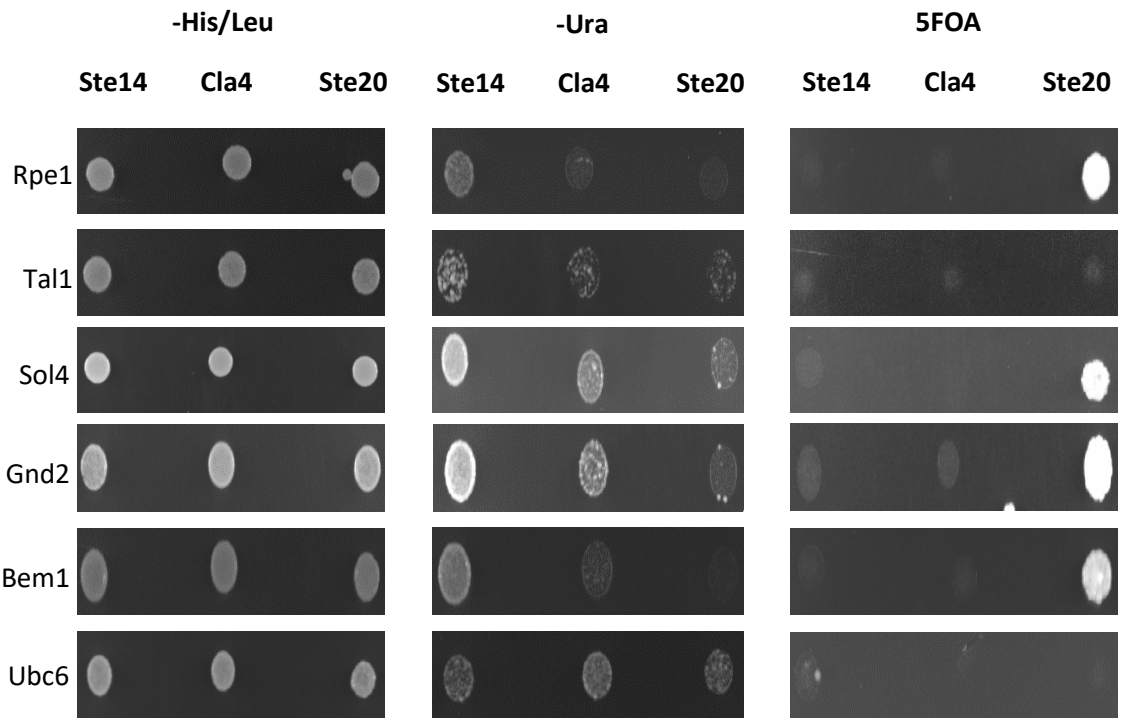


Fig 3.4 Protein-protein interaction between Ste20/Cla4 and enzymes involved in PPP. The indicated strains, Rpe1, Tal1, Sol4, Gnd2, were spotted at a dilution of 1:10 on medium lacking histidine and leucine for growth control while selection for interaction was done on medium lacking uracil and medium supplemented with 5FOA. Cells were grown for 48hours.

Selection was also carried out on plates supplemented with 5FOA and lacking histidine and leucine. Here, only Rpe1, Sol4, Gnd2 and the positive control, Bem1, showed an interaction with Ste20 as growth of cells was observed which indicates Ura3 degradation since the presence of Ura3 alone is sufficient to convert the less toxic 5FOA to the toxic 5 fluorouracil and inversely this means no growth on plates lacking uracil. No growth or interaction was observed for Tal1 with Ste20. No interaction was observed with Cla4 and all four proteins as no growth was observed.

3.3.3 Protein-protein interaction between Ste20/Cla4 and enzymes of glycerol synthesis

Synthesis of glycerol from glucose via dihydroxyacetone phosphate is one of the routes through which glucose is metabolised especially in *S. cerevisiae*. Glycerol serves as the most compatible solute whose role is particularly important in osmoregulation. The role of Ste20 in the regulation of glycerol, via the MAPK cascade, has been well established. In the MAPK cascade, Ste20 is involved in the regulation of gene transcription of enzymes involved in glycerol synthesis under different conditions. For example, under osmotic stress and aerobic condition, *GPD1/GPP2* genes are upregulated while under anaerobic condition, *GPD2/GPP1* are upregulated (Albertyn, et al., 1994; Ansell, et al., 1997; Eriksson, et al., 1995) However, it is yet to be established whether Ste20 and Cla4 interact with these enzymes.

Three genes, *GPD1*, *GPP1* and *GPP2*, encoding corresponding enzymes of glycerol synthesis were amplified using PCR-based approach, cloned in the vector, pADNX as described in section 3.3.1. These proteins were characterised by immunoblotting using western blot and α-HA mouse monoclonal antibody to detect the HA-tag next to the N-ubi of the cloning vector. Plasmids carrying either half of the ubiquitin fused with the Ste20/Cla4 and our genes of interest were transformed in yeast. Transformed yeast cells were spotted on plates lacking histidine and leucine as positive growth control. Selection for interactions was carried out on plates also lacking uracil. Also, plates supplemented with 5FOA and lacking histidine and leucine was used as an alternative.

As shown in fig 3.5, selection was carried out on plates lacking only histidine and leucine as growth control, where all cells grow well due to the presence of *HIS* and *LEU* genes allowing for the synthesis of the amino acids, histidine and leucine, required for cell growth. Selection on plates lacking uracil showed all three proteins, Gpd1, Gpp1 and Gpp2 interact

with Ste20 as no cell growth was observed while Gpd1 and Gpp2 showed strong interaction with Cla4 as no cell growth was observed while Gpp1 showed weak or transient interaction with Cla4 with slight cell growth observed.

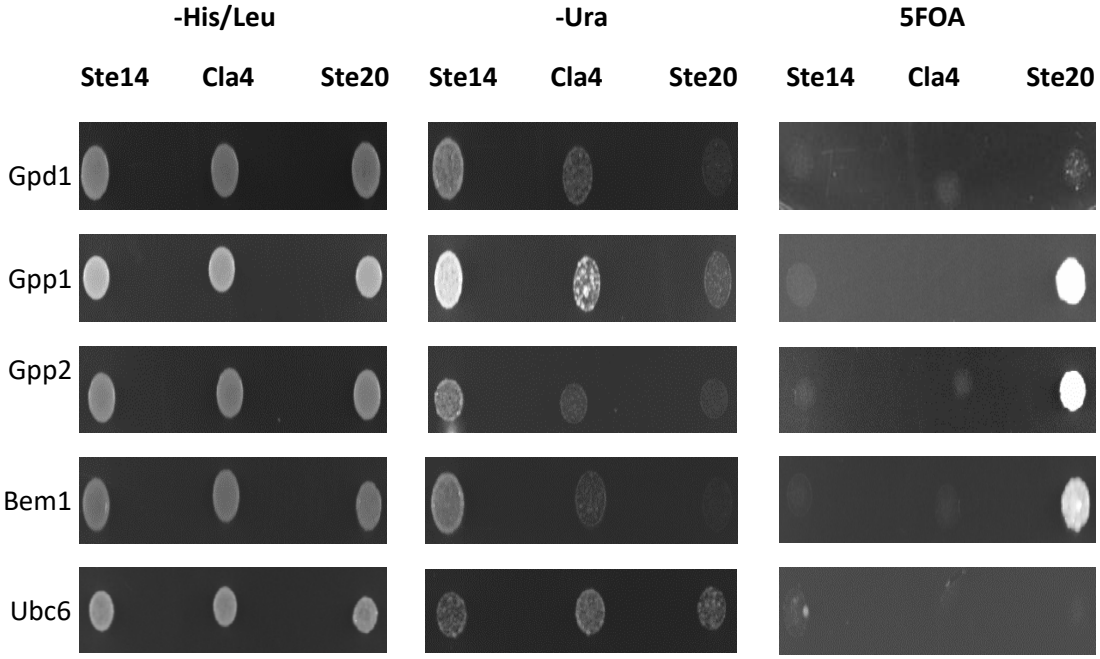


Fig 3.5 Protein-protein interaction between Ste20/Cla4 and enzymes involved in glycerol synthesis. The indicated strains, Gpd1, Gpp1, Gpp2, were spotted at a dilution of 1:10 on medium lacking histidine and leucine for growth control while selection for interaction was done on medium lacking uracil and medium supplemented with 5FOA. Cells were grown for 48hours.

Selection carried out on plates supplemented with 5FOA showed Gpp1, Gpp2 and Bem1, the positive control, interact with Ste20 due to the observed cell growth which indicates Ura3 degradation since the presence of Ura3 alone is sufficient to convert the less toxic 5FOA to the toxic 5 fluorouracil and inversely this means no growth on plates lacking uracil. Gpd1 showed no cell growth with Ste20. All of the tested proteins showed no interaction or cell growth with Cla4.

3.4 Discussion

In this study, the split-ubiquitin system was used to identify an array of proteins involved in the regulation of glucose metabolism. Prior to this work, interaction of proteins involved in glucose metabolism via glycolysis, gluconeogenesis, PPP and glycerol synthesis with Ste20 and Cla4 has not been reported. Ste20 and Cla4 have been shown previously to regulate cellular metabolism such as in sterol biosynthesis where Ste20 was found to interact with Erg4, Cbr1 and Ncp1 involved in sterol biosynthesis using the split ubiquitin system (Tiedje, et al., 2007). It was also shown that Ste20 and Cla4 not only binds to these proteins but also regulate their activity (Lin, et al., 2009). Furthermore, these enzymes were found to play important roles in processes that are regulated by Ste20 such as mating and filamentation suggesting that Ste20 could regulate these processes not only through a change in gene expression via activation of a MAPK cascade but also through modulation of sterol metabolism. Ste20 and Cla4 have also been shown to exert important roles in other aspects of sterol homeostasis such as regulation of proteins that mediate sterol uptake and sterol storage (Lin, et al., 2009). The split-ubiquitin technique was employed in investigating this interaction making it a reliable system for screening of protein interactions.

The study of protein-protein interaction gives an insight into their molecular mechanism. From this study, it was shown that some proteins involved in the glycolytic and gluconeogenic pathways interacted with Ste20 while a few interacted with Cla4. Eno1, Tdh1, Pfk2 and Pck1 showed no growth on plates lacking uracil indicating an interaction with Ste20 while Glk1, Tpi1 and Fbp1 showed a slight growth with Ste20 indicating a weak or transient interaction. Only Eno1, Tdh1, Tpi1 showed weak or transient interaction with Cla4 while others showed no interaction. Proteins involved in PPP were also screened and the

result showed Ste20 and Cla4 interacted with a couple of proteins involved in this pathway. Results showed that Rpe1, Tal1, Sol4 and Gnd2 interacted with Ste20 while Rpe1 and Tal1 showed interaction with Cla4 while a weak interaction was observed with Sol4 and Gnd2.

These data only show a protein-protein interaction. This does not come as a surprise as the closest homologue of Ste20 in mammals, Pak1 has been shown to inhibit glycolysis by regulating phosphoglycerate mutase, PGAM, an enzyme responsible for the interconversion of 3- and 2-phosphoglycerate (Shalom-Barak & Knaus, 2002) and emerging roles of PAKs are becoming evident in glucose homeostasis such as the role of Pak1 in insulin secretion and glucose uptake (Chiang, et al., 2013; Chiang & Jin, 2014).

Finally, the role of Ste20 especially has been well studied in the regulation of glycerol in yeast. Glycerol is an important osmolyte in osmoregulation in yeast. Ste20 has been shown to regulate the synthesis of glycerol via the Hog-MAPK pathway. As much as is known about the role of Ste20 in synthesis of glycerol not much is known about a direct interaction between Ste20/Cla4 and the enzymes involved in glycerol synthesis via dihydroxyacetone phosphate. This study shows Ste20 interacts with Gpd1, Gpp1 and Gpp2 while Cla4 interacts with Gpd1 and Gpp2 while only transiently or weakly interacts with Gpp1. A split-ubiquitin analysis of Gpd2 shows no interaction with both Ste20 and Cla4 (Thomas Hoefken, unpublished data). Two possible explanation can be given to the observed difference in interaction between the two isoenzymes of Gpd, Gpd1 and Gpd2 and not Gpp, Gpp1 and Gpp2. Gpp1 and Gpp2 are quite similar in a number of ways including amino acid sequence identity with around 95% similarity (Norbeck, et al., 1996). Also, Gpp1 and Gpp2 have overlapping functions as seen in their deletion mutant phenotype (Pahlman, et al., 2001). A look at Gpd1 and Gpd2 shows quite a dissimilarity between these two isozymes. Amino acid

sequence identity shows around 69% similarity (Eriksson, et al., 1995). Also, analysis of the promoter region of Gpd1 and Gpd2 shows a very low degree of homology, an indication of difference in regulatory mechanism. This difference in regulatory mechanism is seen in their distinct mutant phenotype where Gpd1 expression is stimulated and increased during growth in aerobic condition while Gpd2 expression is increased during growth under anaerobic condition (Albertyn, et al., 1994). Put together, this could also mean that Ste20 and Cla4 may have a role in processes that involve Gpd1 and not Gpd2, for example in the biosynthesis of glycerol under aerobic conditions but not anaerobic conditions.

However, it is important to understand here that for those proteins showing slight growth in plates lacking uracil, this could be as a result of unspecific protein degradation which has led to the growth of cells (Dirnberger, et al., 2008) but since cell growth were observed in combination with Ubc6, the negative control and not with Bem1, the positive control, these indicates that the observed interaction are quite specific. Screen analysis of the 5-FOA plates showed a few cell growths of protein combinations with Ste20 alone and none with Cla4. A recall that growth on 5FOA indicates the absence of Ura3, an inverse observation of no cell growth in plates lacking uracil while no growth on 5FOA implies the presence of Ura3, which converts less toxic 5FOA to toxic 5fluorouracil, preventing cell growth with an inverse observation of cell growth in plates lacking uracil. For those protein combinations that showed interaction in the plates lacking uracil and not in the 5FOA plates, an explanation to this is that the concentration of 5-FOA used against cell transformants expressing bait-prey interaction is too much for the survival of these cells as 5-FOA concentration is a stringent factor in split ubiquitin screening (Dirnberger, et al., 2008). Another explanation is that plating density - the number of transformed cells per cm^2

medium area – affects the selection pressure by 5-FOA. When the density of transformed cells carrying the R-Ura3 is less than the density of corresponding 5-FOA it exerts a stronger selective pressure and hence decreases the survival rate of transformed cells (Dirnberger, et al., 2008).

Other enzymes of the four-major glucose metabolic pathway, glycolysis, gluconeogenesis, the PPP and glycerol biosynthesis, have now been tested as shown in table 6. Together these data satisfy that all the enzymes have now been tested for interaction. Most of them interact with Ste20 while several also with Cla4. It is therefore tempting to speculate that Ste20 and to some extent Cla4 regulate entire glucose metabolic pathways. This is particularly interesting because key processes regulated by Ste20/Cla4 such as mating, filamentation and hyperosmotic stress response are linked to glucose metabolism and availability. So far, this only suggests the possibility of a direct biochemical effect of Ste20 and Cla4 on these proteins activity and/or possibly other factors such as protein stability. Further studies will be carried out to better understand and investigate the molecular mechanism through which Ste20 and Cla4 regulate these proteins.

It has been shown here that Ste20 and Cla4 interact with a several enzymes involved in glycolysis, gluconeogenesis, the PPP and glycerol synthesis, importantly those involved in glycerol synthesis. Since Ste20 and Cla4 have a well-established role in glycerol biosynthesis and the pathway is quite a simple one with only two reaction steps as compared to others, the remaining aspect of this research will be focused on this link.

Table 6. List of complete protein-protein interactions between Ste20 and Cla4 and enzymes involved in glycolysis, gluconeogenesis, PPP and glycerol synthesis.

Proteins	Ste20	Cla4
Hxk1	-	-
Hxk2	+++	+
Glk1	+++	+
Pgi1	+++	+++
Pfk1	+++	+++
Pfk2	+++	-
Fba1	+++	+++
Tpi1	+++	+++
Tdh1	+++	+++
Tdh2	+++	+++
Tdh3	+++	+++
Pgk1	+++	+++
Gpm1	+++	+++
Eno1	+++	+
Eno2	+++	+++
Cdc19	+++	-
Zwf1	+	-
Sol3	+++	+
Sol4	+++	+++
Gnd1	+++	+++

Gnd2	+++	+++
Rki1	+++	-
Rpe1	+++	+++
Tkl1	+++	+++
Tkl2	+++	+++
Tal1	+++	-
Pck1	+++	-
Fbp1	+	-
Gpd1	+++	+
Gpd2	-	-
Gpp1	+++	+++
Gpp2	+++	+

“+++ = strong interaction” “+ = weak or transient interaction” “- = no interaction”

Chapter 4:

Investigating the effect of Ste20 and Cla4 on the expression and activity of Gpd1 under optimal growth conditions.

4. Investigating the effect of Ste20 and Cla4 on the expression and activity of Gpd1 under optimal growth conditions

4.1 Introduction

Glycerol plays a highly significant role in yeast. Beyond its well-studied role in regulating different stress conditions, importantly hyperosmotic stress, its relevance as an important compatible solute required for growth under different conditions is highly recognised. Under non- stressed conditions, yeast cells still metabolise glycerol for many physiological purposes, such purposes are observed in the role of glycerol synthesis in balancing of redox potentials (Van Dijken & Scheffers , 1986) and the formation of glyceride lipids, a component of cell membrane, from the intermediate of glycerol synthesis, glycerol 3-phosphate (Paltauf, et al., 1992). During growth of yeast cells on glucose, yeast cells undergo glycolysis which produces numerous intermediates, some of which are used in the de novo synthesis of amino acids and other biomolecules and these processes result in the excessive generation of NADH. This imbalance in the redox currency of the cell is unfavourable and dangerous and as such the excess NADH must be re-oxidised to NAD^+ to establish a balance in the NADH/NAD^+ of the cell. The production of glycerol is one way the cell re-oxidises the excess NADH generated (Ansell, et al., 1997). In yeast, glycerol is produced via a two-step enzyme catalysed reaction from dihydroxyacetone phosphate (fig 4.1) through the action of the NADH-dependent glycerol 3-phosphate dehydrogenase (Gpd) and glycerol 3-phosphatase (Gpp).

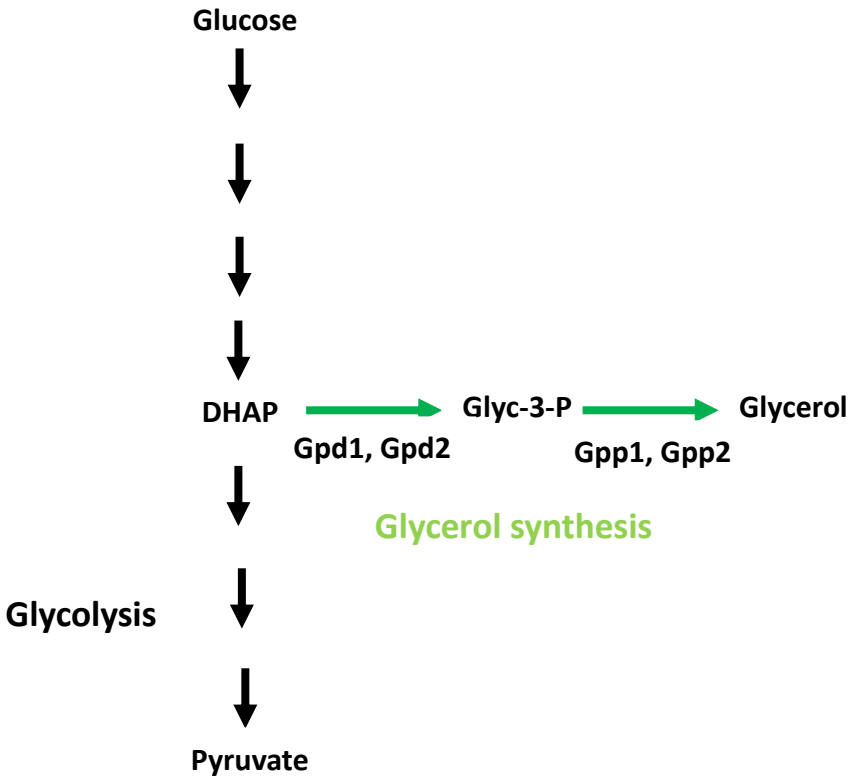


Fig 4.1 Intracellular glycerol synthesis in yeast. Glycolysis (black arrows) supplies dihydroxyacetone phosphate, DHAP, a metabolite from which glycerol is synthesised. Glycerol synthesis from DHAP (green arrows) occurs via two enzymatic steps regulated by glycerol 3-phosphate dehydrogenase (Gpd1 and Gpd2) and glycerol 3-phosphatases (Gpp1 and Gpp2).

Cytoplasmic glycerol 3-phosphate dehydrogenases, Gpds, catalyse the first and rate limiting step in the synthesis of glycerol via an NADH-dependent reduction of dihydroxyacetone phosphate, DHAP, to glycerol 3-phosphate. Gpds mediate a number of cellular processes notably in the aspect of cell growth. They have been implicated in maintaining cell growth under different circumstances and capacities. During cell growth, GPDs regulate cellular redox balance by re-oxidising NADH produced from a number of cellular processes including glycolysis and this is also important for anaerobic growth (Ansell, et al., 1997). Also, GPDs have been reported to reduce the accumulation of the toxic metabolite, methylglyoxal, by disposing off of its precursor DHAP (Aguilera & Prieto, 2004). GPDs play a key role in the synthesis of phospholipids by supplying the precursor molecule glycerol 3-phosphate

(Fraenkel, 2011). Important to mention is the osmoprotective role of GPDs under hyperosmotic stress condition where they contribute to the intracellular accumulation of glycerol (Albertyn, et al., 1994).

In yeast, two isoenzymes of Gpds are known, Gpd1 and Gpd2. *In vivo* studies have revealed that Gpd1 and Gpd2 have partially overlapping functions (Lee, et al., 2012). Under anaerobic conditions, single deletion of *GPD2* but not *GPD1* results in the slow growth of cells (Ansell, et al., 1997) while under hyperosmotic stress condition, single deletion of *GPD1* has been reported to result in osmosensitivity of cells (Albertyn, et al., 1994). Amino acid sequence analysis of Gpd1 and Gpd2 have revealed a 69% identity between them (Eriksson, et al., 1995), one possible explanation for their partial overlapping function. Another possible explanation could be the very low degree of homology found within the promoter regions of Gpd1 and Gpd2 (Scanes, et al., 1998). This explains the distinct localisation and regulation of both Gpd1 and Gpd2.

Gpd1, a 42.8kDa protein, is found within the cytosol and peroxisomes while Gpd2, a 42.3kDa protein, is found within the cytosol and mitochondria. Gpd1 has been reported to be the more relevant of the two isoenzymes under aerobic conditions (Albertyn, et al., 1994). Overexpression of Gpd1 has been reported to increase glycerol levels while overexpression of the Gpd2 has no effect (Pahlman, et al., 2001; Nevoigt & Stahl, 1996). In addition to all of these, Gpd1 and Gpd2 are differentially regulated at the transcriptional level (Lee, et al., 2012). Gpd1 has been reported to be transcriptionally upregulated during hyperosmotic stress and a key target of Hog1, the MAP kinase of the HOG-MAPK pathway (Albertyn, et al., 1994) while Gpd2 is transcriptionally upregulated during conditions of anaerobiosis (Ansell, et al., 1997)

The second enzyme involved in the synthesis of glycerol, glycerol 3-phosphatase, GPP is responsible for the dephosphorylation of glycerol 3-phosphate to glycerol. Two isoenzymes of GPP exist, Gpp1 and Gpp2. Amino acid sequence of the two isoenzymes indicate they share 95% identity (Scanes, et al., 1998) with both having a seeming overlapping functions as the deletion of either has no effect on glycerol synthesis under osmotic stress condition. However, both isoenzymes are differentially regulated as Gpp1 along with Gpd2 are actively upregulated form expressed under non-stress and anaerobic condition while Gpp2 and Gpd1 are upregulated isoform during osmotic and oxidative stress conditions (Pahlman, et al., 2001).

This study focuses on investigating Gpd for the purpose that they catalyse the rate limiting step in the biosynthesis of glycerol from glucose. Furthermore, the effect of Ste20 and Cla4 on Gpd1 and not Gpd2 will be investigated. The rationale behind this comes from the split ubiquitin screen of Gpd1 and Gpd2 which shows the interaction of Ste20 and Cla4 with Gpd1 and not Gpd2 and the understanding that Ste20 via the Hog1-MAPK pathway regulates the transcription and expression of Gpd1.

4.2 Aim

- To investigate the effect of Ste20 and Cla4 on the expression of Gpd1 under optimal growth condition.
- To understand the role of Ste20 and Cla4 in the phosphorylation of Gpd1 under this condition
- To investigate whether the deletion of Ste20 and Cla4 play any significant role in the synthesis of glycerol under this condition.

4.3 Results

4.3.1 Expression of Gpd1 in *Δste20* and *Δcla4* single mutant and a *Δste20 cla4-as3* double mutant strains.

One of the aims of this study is to understand the molecular mechanism through which Ste20 and Cla4 exert their biochemical roles on other proteins in their native state. Here, the expression of Gpd1 is investigated.

Firstly, the level of Gpd1 protein was investigated in strains where *STE20* and *CLA4* are overexpressed. Studies have shown that overexpressed genes could play a major role in the regulation of different processes in the cell such as the inhibition of vacuole inheritance by the overexpression of *STE20* or *CLA4* (Bartholomew & Hardy, 2009). In this study, *STE20* and *CLA4* were overexpressed using a multi-copy plasmid, pRS425, as shown in fig 4.2. Fig 4.2a shows western blot of Gpd1 expression level measured against the housekeeping protein Cdc11. This was done to quantify and normalise Gpd1 protein levels in each lane. Sizes of both Gpd1 and Cdc11 protein were checked against a standard protein ladder to confirm the bands on the blots were for the indicated proteins. No difference was observed in the band levels in both overexpressed strains of *STE20* and *CLA4* when compared to that of the wild type strain. The intensity of each band was measured and Gpd1:Cdc11 ratio obtained as relative abundance of Gpd1 protein. Fig 4.2b shows bar chart of mean ± SD of three independent experiments of Gpd1 relative abundance in overexpressed *STE20* and *CLA4* strains. No significant difference in Gpd1 levels were observed in either overexpressed strains and the wild type strain.

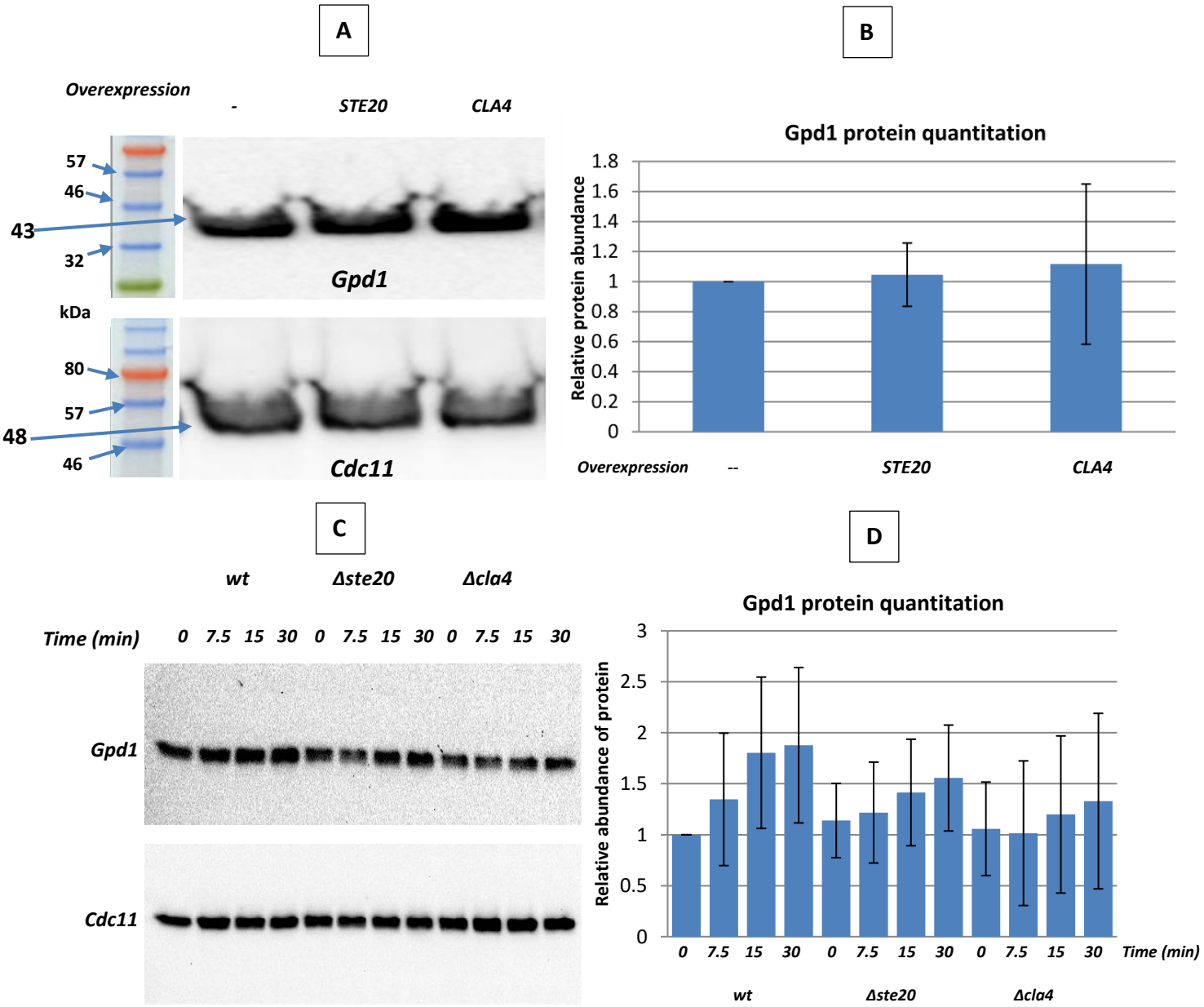


Fig 4.2. The effect of *STE20* and *CLA4* overexpression and *Δste20* and *Δcla4* single deletion on Gpd1 protein level under optimal growth condition in YPD. A) Western blot showing Gpd1 protein levels, quantitated against the housekeeping protein Cdc11, in yeast strains where *STE20* and *CLA4* are overexpressed using the multicopy plasmid pRS425. Protein ladder indicate the bands represent Gpd1 and Cdc11 proteins with sizes 43kDa and 48kDa respectively. B) Bar chart showing mean ± SD of relative abundance of Gpd1 in strains where *STE20* and *CLA4* are overexpressed. C) Western blot showing Gpd1 expression levels quantitated against Cdc11 protein at different time points in *Δste20* and *Δcla4* single deletion strains. D) Bar chart showing mean ± SD of relative abundance of Gpd1 in *Δste20* and *Δcla4* single deletion strains at different time points. Each experiment was performed at least three times independently.

Levels of Gpd1 protein were then investigated in a single deletion strain of *Δste20* and *Δcla4* as shown in fig 4.2c-d. Fig 4.2c shows western blot of Gpd1 protein levels at different time

points measured against Cdc11. The reason behind measuring kinetic assay was due to a preliminary study in the wild type strain that showed that levels of Gpd1 increased with time. Band intensity at time 0 were similar in both single deletion strains and the wild type. At 7.5mins, band intensity in *Δste20* and *Δcla4* strains were slightly reduced compared to those in the wild type while at 15mins intensity of band in *Δste20* were similar to those in the wild type while that for *Δcla4* was slightly reduced. At time 30mins, band intensity in both deletion strains were similar to that in the wild type.

To understand and interpret whether these slight changes indicated a significant decrease, Gpd1 levels at each indicated time point and the corresponding Cdc11 protein were measured. Gpd1:Cdc11 ratio was obtained for each lane and gave the relative abundance of Gpd1 protein. Fig 4.2d shows bar chart of mean ± SD of relative abundance of Gpd1 protein. The data showed that levels of Gpd1 increased considerably in the wild type from 0min to 30mins. In the *Δste20* strain, levels of Gpd1 also increased from 0min to 30mins and these levels were similar to those in the wild type. Levels in the *Δcla4* strain also increased from 0min to 30mins but were slightly lower than those in the wild type. This decrease was however insignificant. These data put together shows that Gpd1 levels are similar and unchanged in both *Δste20* and *Δcla4* strains. This implies that deletion of either *STE20* or *CLA4* has no effect on Gpd1 expression. The result obtained is not surprising as it has been widely reported the redundancy of Ste20 and Cla4 indicating both have an overlapping function (Cvrckova, et al., 1995; Weiss, et al., 2000; Kumar, et al., 2009). Since Gpd1 levels were unchanged in *Δste20* and *Δcla4* single deletion strains, it is necessary to investigate Gpd1 expression levels in a single strain of yeast where both *STE20* and *CLA4* are deleted.

Ste20 and Cla4 have been reported to play key roles in cell viability as single mutant strains of either, *Δste20* and *Δcla4*, remains viable while deletion of both in a single strain is lethal (Cvrckova, et al., 1995). To achieve a viable construct in which both *STE20* and *CLA4* are mutated, several conditional mutants have been described for Cla4 such as the temperature-sensitive *cla4-75* allele, *cla4-75* with a temperature-sensitive degron domain, *cla4-td,* attached to it which makes it degradable at 37^{o}C and the analogue-sensitive *cla4-as* alleles. For both temperature-sensitive alleles, temperature needs to be elevated which could lead to more secondary effects within the cell as this has been reported (Weiss, et al., 2000) . The cla4-td allele has also been reported to carry additional mutations.

For this study, the well-characterised analogue sensitive *cla4-as* allele, *cla4-as3*, was chosen. *cla4-as3* plasmid was constructed and inserted into the *Δste20* strain to replace the wild type *CLA4* as reported by Weiss, et al., 2000. The kinase activity of the Cla4 sensitive analogue allele, *cla4-as3*, is chemically inhibited by 1NM-PP1, (4-amino-1-tert-butyl-3-(1-napthylmethyl)pyrazolo[3,4-d]pyrimidine), a carefully engineered cell-permeble kinase inhibitor that does not affect the wild type kinase only the specified mutant kinase (Bishop, et al., 1998).

To confirm the *cla4-as3* construct, amino acid sequence of the cloned construct was analysed and compared with the wild type kinase as shown in fig 4.3a. Methionine and threonine, two bulky amino acids within the kinase domain of Cla4 are substituted with a smaller amino acid in alanine as reported by Weiss, et al., 2000. One distinct characteristic of cells with a defective or mutant Cla4 kinase is bud elongation (Cvrckova, et al., 1995). This knowledge was used to confirm both the insertion of *cla4-as3* in the *Δste20* strain and the effect of 1NM-PP1 on inhibition of the kinase activity of the analogue allele. As shown in

fig 4.3b-f, wild type cells grown in YPD were treated without and with 10µM 1NM-PP1 and grown for 2hrs after which microscopic analysis was done and image taken (fig 4.3b,c). The image shows that wild type kinase was not affected by 1NM-PP1 as no abnormal bud elongation was observed in those treated with 1NM-PP1 (fig 4.3c) and appeared similar to those cells untreated with the kinase inhibitor (fig 4.3b).

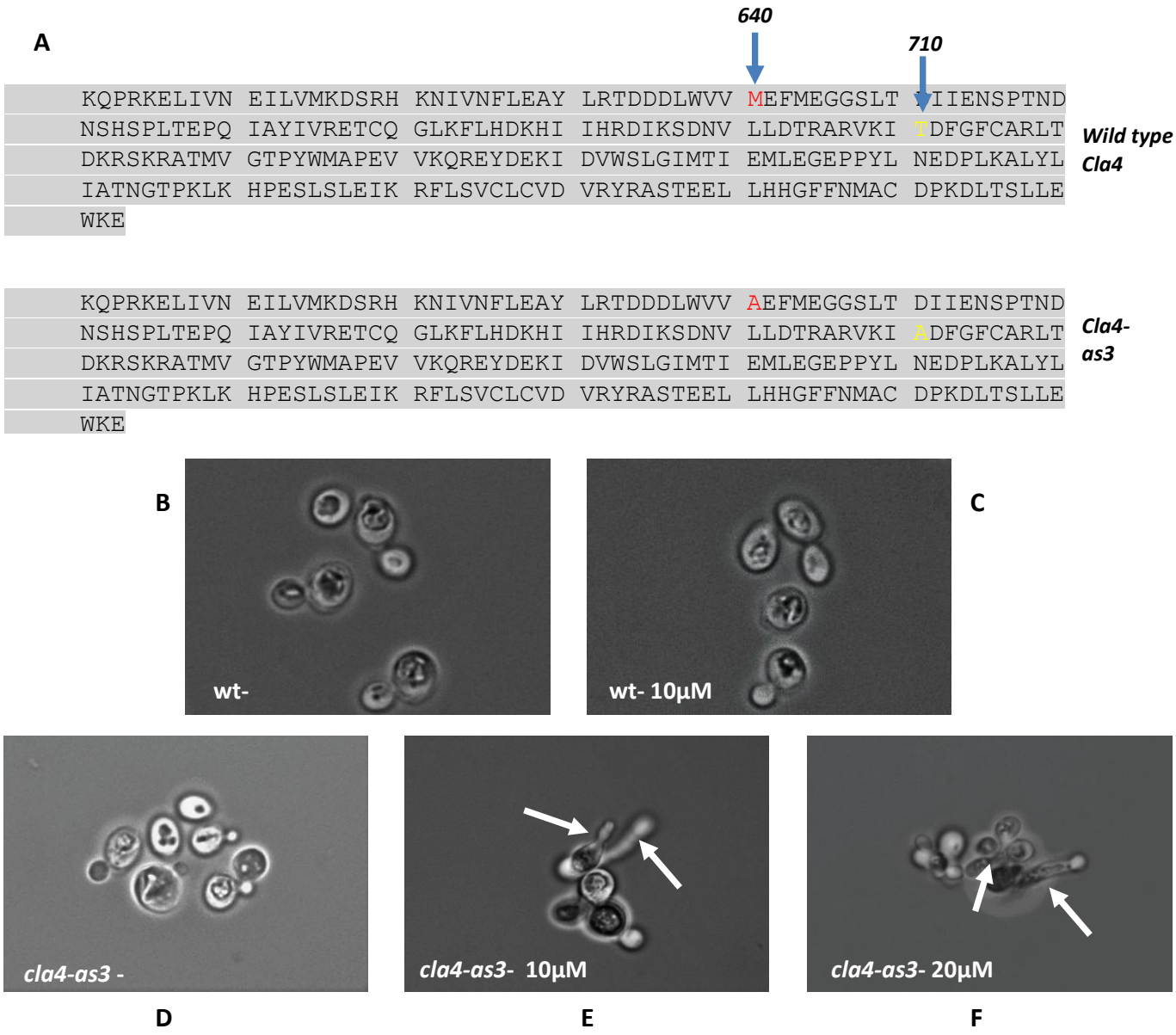


Fig 4.3 Construction of analogue sensitive Cla4 allele, cla4-as3 and microscopic analysis of cell morphology treated with the kinase inhibitor 1NM-PP1. A) Sequence analysis showing positions where amino acids have been substituted from the wild type and the analogue sensitive allele. B and C) Shows normal cell morphology of wild type treated without, wt-, and with 10µM 1NM-PP1 respectively. D – F) Cell morphology of *Δste20*, expressing *cla4-as3* allele without 1NM-PP1 and with 10 and 20µM 1NM-PP1 respectively. Bud elongation characterise the defect in Cla4 kinase (white arrows).

Δste20 cells carrying *cla4-as3* allele (for convenience this will be referred to as *Δste20 cla4-as3)* were then analysed under different exposure condition to 1NM-PP1. In fig 4.3d, *Δste20 cla4-as3* was untreated with 1NM-PP1 and a similar morphology, round buds, to that of the wild type was observed indicating the analogue allele retains the characteristics of the wild type kinase. However, when treated with 1NM-PP1 at concentrations of 10µM and 20µM, an aberrant cell morphology characterised by elongated buds (white arrows) was observed in the *Δste20 cla4-as3* strain (fig 4.3e,f). For every experiment performed, 10µM was used as it has been well reported and shows the expected morphological defect.

Levels of Gpd1 protein were investigated in the *Δste20 cla4-as3* strain treated with 1NM-PP1. Fig 4.4a shows western blot of Gpd1 expression levels in the wild type and *Δste20 cla4-as3* strains at different time points measured against Cdc11 for protein normalisation. Gpd1 expression level in the wild type strain increased from 0min to 30mins while in the *Δste20 cla4-as3,* expression levels were reduced at 0min and remained unchanged till 15mins with a slight increase at 30mins. To analyse the observed expression of Gpd1 in both strains, Gpd1:Cdc11 ratio was obtained and given as the relative abundance of Gpd1 protein. Relative abundance of Gpd1 expression was then represented as mean ± SD on a bar chart with the p-value represented as $p<0.02$. Fig 4.4b shows level of Gpd1 in the wild type increased from 0min to 30mins while levels of Gpd1 in *Δste20 cla4-as3* were significantly lowered, with about a two-fold reduction at 0min which goes to about a four-fold reduction at 30mins when compared to those in the wild type. Another important observation in the *Δste20 cla4-as3* strain shows that Gpd1 levels were relatively unchanged at all indicated time points.

Based on the lower levels of Gpd1 protein observed in the *Δste20 cla4-as3* strain, it was necessary to investigate whether these changes are due to a loss of induction of transcription via Ste20/Cla4-Ste11 link of the MAPK cascade or other unknown mechanism. Gpd1 levels were analysed in *Δste11* single deletion strain. Western blot image, fig 4.4a, showed that Gpd1 levels increased from 0min through to 30mins and a difference in protein expression was observed when compared to the *Δste20 cla4-as3* strain. A look at the bar chart showing the relative abundance of Gpd1 in *Δste11* single deletion strain, fig 4.4b, shows that levels of Gpd1 were significantly increased, about a three-fold increase at 0min through to 30mins, when compared to those in the *Δste20 cla4-as3* strain. furthermore, Gpd1 levels at all indicated time points in the *Δste11* strain were similar to those in the wild type.

Based on the outcome of this experiment, it is conceivable that the absence of Ste20 and Cla4 activity probably inhibited the transcription or translation of *GPD1*. However, results of Gpd1 levels observed in *Δste20 cla4-as3* strain showed a relatively unchanged expression level from 0min to 30min which would be expected to reduce continuously should transcription or translation of *GPD1* be inhibited. Ruling out the possibility of inhibition of transcription or translation as the reason for the lower levels of Gpd1 observed in *Δste20 cla4-as3* strain, the only other factor that affects protein level and in this case, is protein stability.

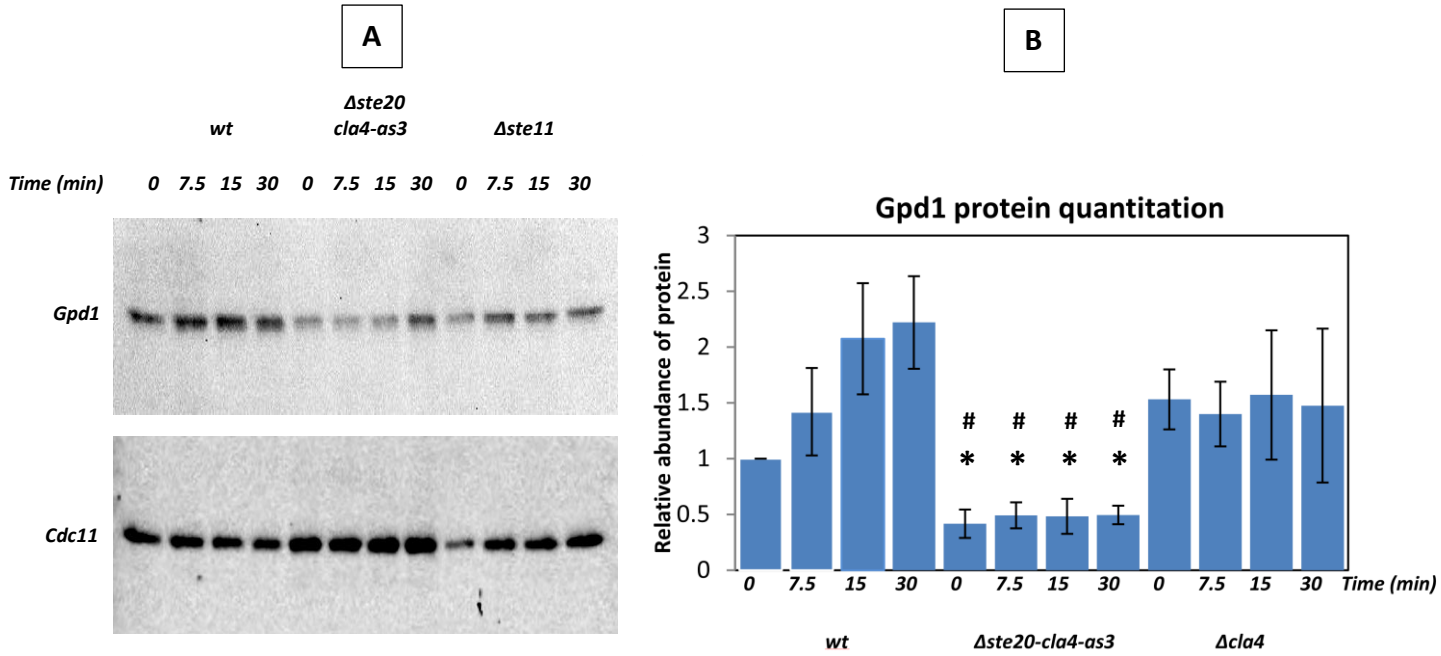


Fig 4.4. Effect of Δste20 cla4-as3 double mutation on Gpd1 expression level. A) Western blot showing kinetic assay of Gpd1 levels in wt, *Δste20 cla4-as3* and *Δste11* strains under optimal growth condition in YPD medium and quantitated against the housekeeping protein, Cdc11. B) Bar chart showing mean ± SD kinetic assay of relative abundance of Gpd1 protein in all indicated strains under optimal growth condition. Each experiment was conducted at least three times independently. P-values, p<0.05, are determined using the one-way anova statistics tool. *p<0.02 shows significant difference between wt and *Δste20 cla4-as3* while #p<0.02 shows significant difference between *Δste11* and *Δste20 cla4-as3.*

4.3.2 Phosphorylation status of Gpd1 in *Δste20 cla4-as3* double mutant strain

PAKs, mediate their biochemical effects by transferring phosphate group(s) on either serine or threonine amino acid residues. Phosphorylation is a post-translational modification process that regulates the biochemical activity, subcellular localization and stability of proteins (Olsen, et al., 2006). In this study, the phosphorylation status of Gpd1 was investigated in a double mutant strain. The rationale behind this investigation is due to the fact that Gpd1 protein has been reported as a phosphoprotein regulated by a number of kinases in yeast under different conditions (Lee, et al., 2012; Oliveira, et al., 2012).

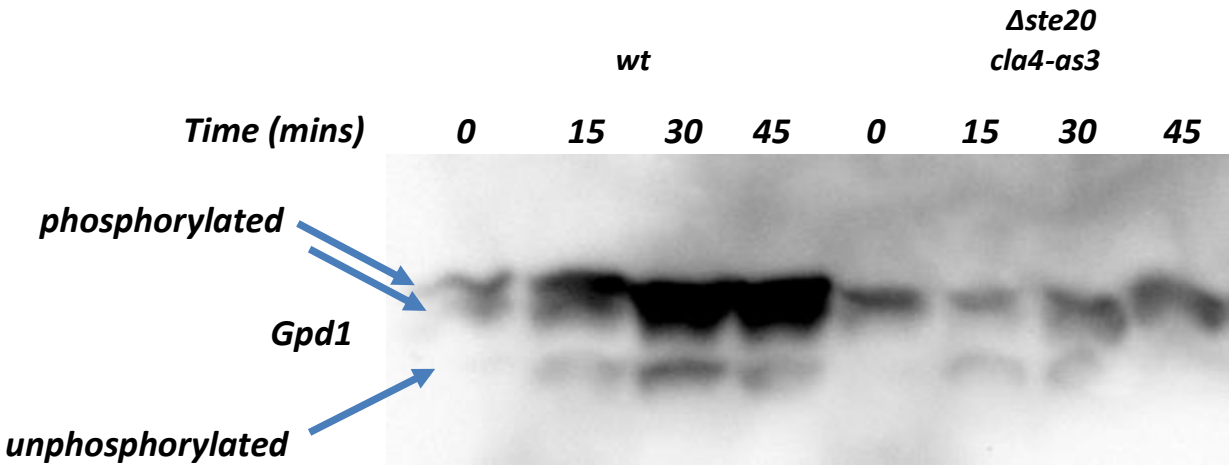


Fig 4.5 Phos-tag SDS-PAGE showing the phosphorylation status of Gpd1. Phosphorylation status of Gpd1 protein is investigated in both wild type and a *Δste20 cla4-as3* double mutant strain grown in YPD. Kinetic assay was performed on exponentially growing cells to investigate changes in the level of phosphorylated proteins. Three bands are shown here, top band represents di-phosphorylated proteins, middle band; mono-phosphorylated proteins and the lowest band; unphosphorylated proteins

To detect phosphorylated protein, a phosphate affinity Phos-tag SDS-PAGE which operates by retarding the mobility of phosphoproteins (Kinoshita, et al., 2006) was used. The Phos-tag SDS-PAGE operates on the principle of differences in migration of proteins based on molecular weight. When the Phos-tag binds to phosphorylated proteins it causes a shift in their migration in the gel settling above the unphosphorylated proteins. The more

phosphorylated the protein, the slower it migrates and settles above lesser and non-phosphorylated proteins.

Here, fig 4.5 shows the phosphorylation status of Gpd1 protein in both wild type and *Δste20 cla4-as3* double mutant strains. Kinetic assay was performed to monitor the changes in the level of phosphorylated proteins. Cells were grown in YPD medium to an OD_{600} of 0.5 for about 3hrs. Both wild type and *Δste20 cla4-as3* strains were treated with 10µM 1NM-PP1. Phosphorylation status of Gpd1 in the wild type showed there is a remarkable increase in the levels of phosphorylated species from time point 0 indicated by the intensity of bands on the blot. Two important observations are evident here, firstly, three layers of bands can be observed with the two upper bands representing di- and mono-phosphorylated proteins respectively. This shows that Gpd1 can be both mono and di-phosphorylated. Secondly, ratio of phosphorylated bands to un-phosphorylated bands increases greatly indicating that under optimal growth condition, a large number of Gpd1 existed in the phosphorylated form. In the double mutant *Δste20 Δcla4-cla4-as3* strain, levels of phosphorylated species were remarkably reduced at all indicated time points when compared to those of the wild type strain and this level remained relatively constant. At time point 0, no unphosphorylated protein was observed as most of the expressed proteins were phosphorylated and this was similar to that of the wild type. At time points 15 and 30, levels of unphosphorylated proteins slightly increased but considerably reduced in comparison to those of the wild type while at 45 mins, very little unphosphorylated protein was observed and considerably reduced in comparison to that in the wild type. This shows that deletion of Ste20 and Cla4 in a single strain affected the expression of phosphorylated specie of Gpd1.

4.3.3 Levels of intracellular and extracellular glycerol in both *Δste20* and *Δcla4* single deletion and *Δste20 cla4-as3* double mutant strains.

Since Gpd catalyse the rate limiting step in the synthesis of glycerol and the effect of Ste20 and Cla4 have been investigated in the expression of Gpd1 and its phosphorylation status, it is important to look at the effect they might have on synthesis and/or overall accumulation of glycerol. Here, the effect of Ste20 and Cla4 on glycerol synthesis and accumulation will be investigated.

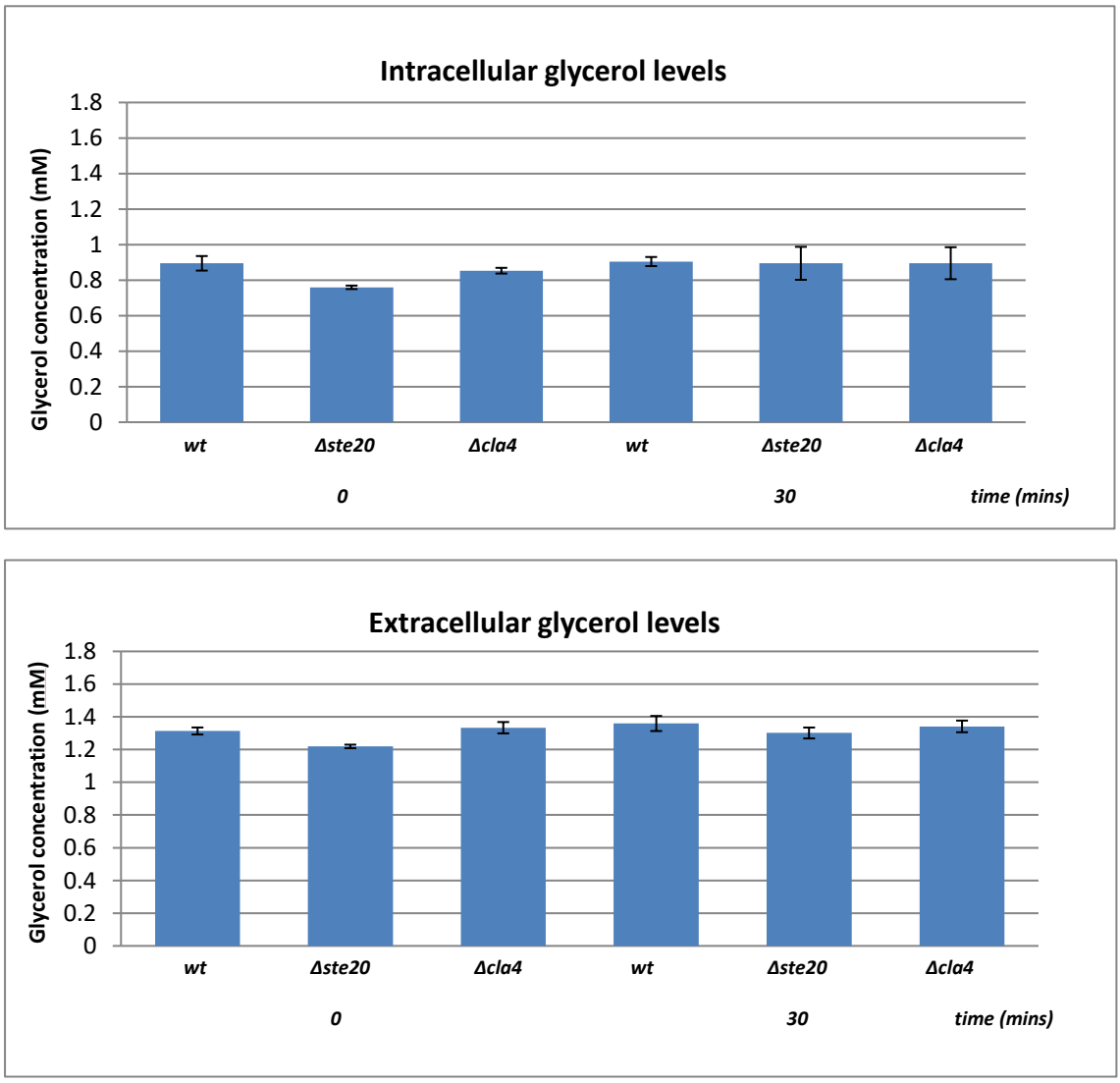


Fig 4.6 Effect of Ste20 and Cla4 on the synthesis and accumulation of glycerol. Levels of glycerol were measured intracellularly and extracellularly in *Δste20 and Δcla4* single deletion strains at the indicated time points. Each experiment was conducted as independent triplicates.

Levels of glycerol were measured in single deletion *Δste20 and Δcla4* strains. Cells were grown in YPD medium to an OD_{600} of 0.5 for about 3hrs. Levels of both intracellular and extracellular glycerol were unchanged in the single deletion *Δste20 and Δcla4* strains when compared to the wild type strain, fig 4.6. This shows single deletion of either PAKs have no effect on accumulation and synthesis of glycerol.

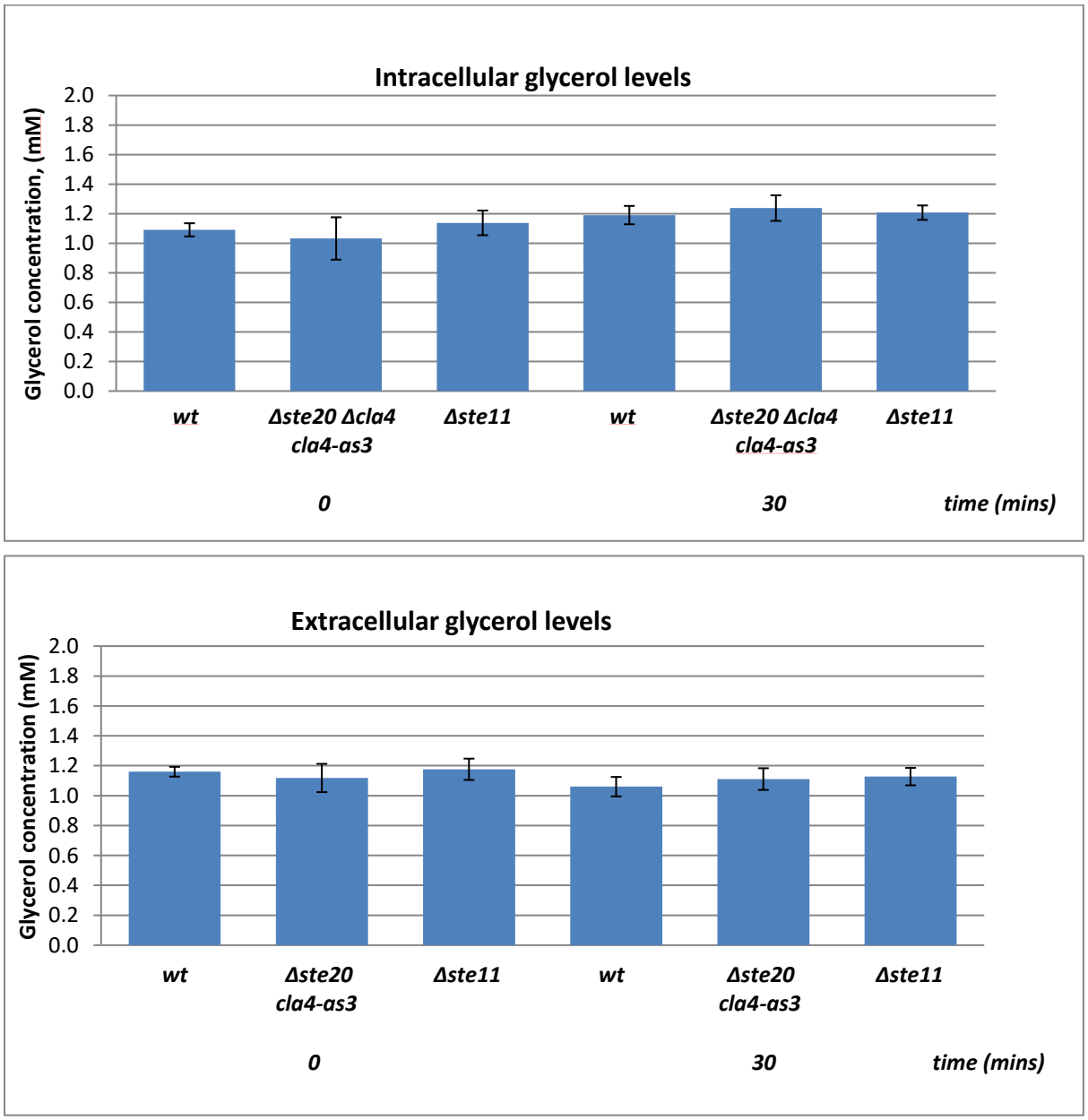


Fig 4.7 Effect of double mutant *Δste20 Δcla4-cla4-as3* on synthesis and accumulation of glycerol. Levels of intracellular and extracellular glycerol were measured in the double mutant *Δste20 Δcla4-cla4-as3* and *Δste11* at the indicated time points. Each experiment was conducted at least three times independently.

Also, levels of intracellular and extracellular glycerol were measured in double deletion *Δste20 cla4-as3* strain and in the *Δste11* strain. The rationale behind this is if there is an effect for double mutation of Ste20 and Cla4 it is necessary to find out whether this is due to transcriptional control or other mechanisms. Fig 4.7 shows that the levels of glycerol, intracellularly and extracellularly, remained unchanged in both strains and were similar to those in the wild type. This shows deletion of Ste20 and Cla4 has no effect on the synthesis and accumulation of glycerol.

4.4 Discussion

The role of Ste20 and Cla4 in gene expression through MAPK is well established (Chen & Thorner, 2007). Since Ste20 and Cla4 have been shown to bind to Gpd1 it was essential to examine whether there is a direct interaction in terms of regulation of Gpd1. The aim of this study was to investigate and understand the mechanism of action of Ste20 and Cla4 in the regulation of glycerol biosynthesis by looking at how they regulate the expression of Gpd1, its post-translational modification status i.e. phosphorylation status and finally the synthesis of glycerol. Ste20 and Cla4 have been shown to be involved directly in the MAPK pathway in the regulation of gene expression of *GPD1*, where they phosphorylate a downstream MAPKKK protein, Ste11. In this study, to understand the direct relationship Ste20 and Cla4 has on the regulation of Gpd1 expression, expression of Gpd1 was simultaneously investigated in a *Δste11* strain. The hypothesis for this study was that since Ste20 and Cla4 act directly on Ste11 in the MAPK pathway, the same effect of expression of Gpd1 should be observed in the *Δste20 cla4-as3* double mutant strain and the *Δste11* strain. Investigating the effect of double deletion of *STE20* and *CLA4* is impossible as deletion of both kinases is lethal to the cell. To achieve the double deletion effect of *STE20* and *CLA4*, a *CLA4* analogue-sensitive allele, *CLA4-as3* was used and transformed in a *Δste20* background. The results obtained show that the expression of Gpd1 in *Δste11* strain was similar to that of the wild type and this effect was the same for the single mutant *Δste20* and *Δcla4* strain - this observation is consistent with the fact that Ste20 and Cla4 have overlapping functions (Cvrckova, et al., 1995). However, expression of Gpd1 in the double mutant strain was significantly reduced when compared to that of the wild type and *Δste11* strain. This shows that Ste20 and Cla4 have an effect on the expression of Gpd1 protein under optimal growth

condition but does not explain in what way or how it regulates the expression. Intracellular levels of proteins are determined by the rate of synthesis and rate of degradation or better put, overall protein turnover (Callis , 1995). Since Gpd1 levels are not reduced in the *Δste11* cells in which there is no regulation of transcription by Ste20 and Cla4, the data obtained here suggest that the reduced level of Gpd1 observed in *Δste20 cla4-as3* is due to degradation of Gpd1 rather than the inhibition of transcription – as it is expected with transcriptional inhibition, protein synthesis should reduce considerably which is not what was observed here.

Also, the phosphorylation status of Gpd1, a multi-phosphoprotein (Oliveira, et al., 2012), was investigated as it is known that Ste20 and Cla4 are both kinases and mediate their physio-biochemical role by transferring a phosphate group or groups to specific amino acid residues, in their case serine and threonine residues. Phosphorylation of proteins, a post translational modification (PTM) process, is one of the most common and widespread forms of PTM in yeast (Bodenmiller & Aebersold , 2010). Also, phosphorylation of proteins is known to regulate the activity several enzymes of yeast central carbon metabolism (Oliveira & Sauer, 2012). Protein phosphorylation mediate cellular processes where they regulate proteins by activation or inactivation, localisation and marking or unmarking of protein for degradation. In the case of Gpd1, several proteomics studies have shown that phosphorylation of Gpd1 inhibits its activity and four phosphosites, S23, S24, S25 and S27 have been identified (Oliveira & Sauer, 2012). In the wild type grown in glucose, S24 and S27 have been identified as the phosphorylated site and Gpd1 can either be singly or doubly phosphorylated with either forms of phosphorylation and phosphosite sufficient enough to inhibit Gpd1 activity (Oliveira & Sauer, 2012). Here, it is shown that in the absence of both

Ste20 and Cla4, Gpd1 remains multi-phosphorylated albeit the amount of phosphorylated species remains highly reduced. This observation differs from what is obtained in the wild type where phosphorylation of Gpd1 increased with time. Studies have shown that several kinases such as Yck1 and Yck2, TORC2- dependent kinases, phosphorylates Gpd1 (Lee, et al., 2012). This probably accounts for the phosphorylation of Gpd1 in the observed in the *Δste20 cla4-as3* strain. Taken together, this shows that Ste20 and Cla4 affects the level of Gpd1 expression and this affects the phosphorylation level but does not prevent the phosphorylation of Gpd1.

Finally, levels of glycerol were measured as it is already observed that Gpd1 stability and expression is greatly reduced in the double mutant strains and not in the single mutant strain. The idea behind measuring extracellular levels of glycerol was that overall glycerol levels might be affected by manipulating Ste20 and Cla4 and this might be missed if only intracellular levels were measured. The data obtained showed no difference in the levels of glycerol in the single mutant, *Δste20* and *Δcla4* strain, double mutant *Δste20 Δcla4-cla4-as3* strain and *Δste11 strain.* This also suggests that the synthesized Gpd1 protein in the double mutant strain is sufficient to produce glycerol at the same level as seen in the wild type. It has been reported that only in *Δgpd1* strain is glycerol level and Gpd1 activity reduced by a significant amount when grown in glucose under aerobic condition (in this case, optimal growth condition) where the second isoform of Gpd, Gpd2, plays a significant role (Oliveira, et al., 2012).

Chapter 5:

Investigating the effect of Ste20 and Cla4 on the expression and activity of Gpd1 under hyper-osmotic stress.

5. Investigating the effect of Ste20 and Cla4 on the expression of Gpd1 under hyper-osmotic stress.

5.1 Introduction

Living cells constantly are faced with different kinds of stress and hence the need to develop mechanisms to overcome these stressful situations. Some of the well-studied stress conditions are heat shock, oxidative stress and hyper-osmotic stress. In the yeast cell, hyperosmotic stress is a common condition characterised by the massive efflux of water from the cytoplasm resulting in the shrinkage and loss of cell turgor. This results in the sudden increase in the concentration of macromolecules and possibly among other things, the collapse of the cytoskeletal structure of the cell leading to the depolarization of actin patches (Mager & Siderius, 2002). Yeast cells have developed a unique way of overcoming this stress condition through the use of compatible solutes such as xylitol, arabitol, mannitol and importantly glycerol, an organic polyol (Scanes, et al., 1998). Glycerol accumulation and other compatible solutes have been shown to have no appreciable effect on enzyme function (Brown, 1978). During osmoregulation in yeast, several molecular events are triggered (Mager & Siderius, 2002). To start with, studies have shown that cells temporarily arrest growth suggestively with the aim of re-adjusting their mechanism to accommodate for the sudden change in osmotic status. The point of cell cycle that indicates this arrest is yet unclear as two points within which this arrest can occur have been reported. Two separate studies have shown a block in cell cycle at the G1 and G2/M phase with a down regulation of the Cln3p-Cdc28p kinase activity (Belli, et al., 2001) and inhibition of Clb2p-Cdc28p kinase (Alexander, et al., 2001), respectively. Another important molecular event triggered is the activation of the HOG-MAPKinase pathway. This pathway is characterised by

the activation of Hog1, a homologue of p38 in mammals (Gacto, et al., 2003). Activation of the HOG-MAPK cascade is controlled by two distinct transmembrane osmosensors, Sln1 and Sho1. Inhibition of Sln1 during osmotic stress results in its dephosphorylation and its interaction with a MAPKKKK, Ssk1. Ssk1 then binds and activate Ssk2/Ssk22 complex, a MAPKKK, which in turn activates the MAPKK scaffold protein, Pbs2. Pbs2 then phosphorylates and activates Hog1. Downstream of the second osmosensor, Sho1, lies the GTPase activated PAKs, Ste20 and Cla4. Ste20/Cla4 activates Ste11, a MAPKKK, which then binds and activates Pbs2.

Hog1 plays a crucial role in the diversion of glycolytic flux towards glycerol synthesis, as well as its uptake and efflux, during osmoadaption (Babazadeh, et al., 2017). In adaptation to osmotic stress, Hog1 regulates the transcription of a number of genes such as and importantly *GPD1*, the active isoform of *GPD* under this condition and *GPP2* (Albertyn, et al., 1994; Eriksson, et al., 1995; Babazadeh, et al., 2014). An important event worthy of mention during response to osmotic stress is the transient repression of protein synthesis as it has been shown that Hog1 activates Rck2p, a putative cytoplasmic calmodulin protein that inhibits protein biosynthesis (Bilsland-Marchesan, et al., 2000).

5.2 Aim

- To investigate the effect of Ste20 and Cla4 on the expression of Gpd1 under hyper-osmotic stress condition.
- To understand the role of Ste20 and Cla4 on the phosphorylation status of Gpd1 under hyper-osmotic stress condition.
- To investigate whether the deletion of Ste20 and Cla4 play any significant role in the synthesis of glycerol under this condition.

5.3 Results

5.3.1 Expression of Gpd1 in single deletion strain of *STE20* and *CLA4* and in a double mutant strain.

Here, the effect of Ste20 and Cla4 on expression level of Gpd1 is investigated under high osmotic stress condition. Response to osmotic stress in yeast has been well studied and Ste20 in particular has been identified to play a key role in the regulatory HOG-MAPK pathway that predominantly induces Gpd1 expression for the increased and sustained synthesis of glycerol required to transiently balance the osmotic pressure experienced by affected cells. No data has however identified the direct relationship between Gpd1 and Ste20/Cla4 beyond the point that they activate Ste11, a MAPKKK, which through a series of further activation downstream the HOG-MAPK cascade induce the transcription of *GPD1*. Here, the direct relationship Ste20 and Cla4 exert on Gpd1 under osmotic stress condition is investigated.

Expression of Gpd1 was first examined in *Δste20 and Δcla4* single mutant strains. Cells were treated with 0.8M NaCl to induce osmotic stress and kinetic assay were performed at different time points to monitor changes in the level of Gpd1. Fig 5.1a shows western blot of Gpd1 expression measured against Cdc11, a housekeeping protein. Based on observation in the wild type, level of Gpd1 slowly increased from time point 0, point of osmotic stress stimulation, to 7.5mins. At 15mins, Gpd1 level increased considerably and this further increased greatly at 30mins. This expression pattern suggests a strong induction of Gpd1 after about 15mins of osmotic stress stimulation. Same expression pattern was observed in both *Δste20* and *Δcla4* cells at each time point as those in the wild type. Gpd1:Cdc11 ratio was obtained to normalise protein levels in each lane and this was represented as the

relative abundance of Gpd1 protein. Fig 5.1b shows bar chart of relative abundance of Gpd1 levels in wild type, *Δste20* and *Δcla4* strains. In the wild type, levels of Gpd1 slowly increased from 0min to 7.5mins while this increased about two-folds at 15mins and a further two-fold increase at 30mins. This expression pattern indicates a strong induction of Gpd1 in response to osmotic stress. This shows that Gpd1 is considerably upregulated during response to osmotic stress in budding yeast. In *Δste20* and *Δcla4* cells, same pattern as well as similar levels of expression of Gpd1 was observed at each corresponding time points with those in the wild type. This finding suggests that either Ste20 and Cla4 alone are not essential for osmoadaptation.

Expression level of Gpd1 was then investigated in a *Δste20 cla4-as3* double mutant strain. Fig 5.1c shows western blot of Gpd1 expression measured against Cdc11 at different time points. In the wild type, intensity of bands was relatively unchanged after 5mins of osmotic stress induction which rapidly increased at 10mins and this increase in band intensity continued at 15mins and 20mins. This rapid increase in Gpd1 band observed at 10mins up till 20mins is an indication of a strong induction of Gpd1 expression under osmotic stress. In the *Δste20 cla4-as3* cells, intensity of Gpd1 band was relatively unchanged from 0min to 10mins after osmotic stress induction and this slightly increased at 15mins and a considerable increase at 20mins. When compared to the pattern of expression in the wild type, a delay in Gpd1 expression is observed in the *Δste20 cla4-as3* double mutant strain. To normalise Gpd1 levels, Gpd1:Cdc11 ratio was obtained and taken as the relative abundance of Gpd1 protein. Fig 5.1d shows bar chart of relative abundance of Gpd1 levels and in the wild type, Gpd1 levels were slightly increased from 0min to 5mins after which Gpd1 level increased by more than two-folds at 10mins with a considerable increase at 15mins and

20mins. A strong induction of Gpd1 is observed between 5 and 10mins. In the *Δste20 cla4-as3* double mutant strain, Gpd1 levels were relatively unchanged from 0min to 10mins and only slightly increased at 15mins. However, at 20mins Gpd1 levels considerably increased by two-folds. At 10mins where a strong induction of Gpd1 was observed in the wild type, levels of Gpd1 were significantly reduced by more than a two-fold in *Δste20 cla4-as3* cell. At 15mins, a further significant decrease of more than two-folds was observed with a further significant decrease at 20mins. Put together, this data indicates a significant reduction in Gpd1 levels when Ste20 and Cla4 kinase activity are lost in a single strain. Also, since Gpd1 expression level increased slightly in the *Δste20 cla4-as3* double mutant strain, albeit not to the same level in the wild type, it is conceivable to suggest that loss of Ste20 and Cla4 kinase activity in a single strain contributes to the delay of Gpd1 expression under osmotic stress.

Due to the observed decrease and delay in expression of Gpd1 in *Δste20 cla4-as3* double mutant strain, it is necessary to investigate whether this is due to a loss of induction of transcription via Ste20/Cla4-Ste11 link of the HOG-MAPK cascade. Gpd1 levels were investigated in a *Δste11* single deletion strain. Western blot, fig 5.1c, shows similar Gpd1 expression pattern to those of the wild type at each time point and bar chart, fig 5.1d, shows similar Gpd1 levels to those in the wild type at each time point. This suggests that the significant decrease Gpd1 levels observed in the *Δste20 cla4-as3* double mutant strain is not due to a loss of transcription via Ste20/Cla4 and Ste11 but rather stability of Gpd1 protein.

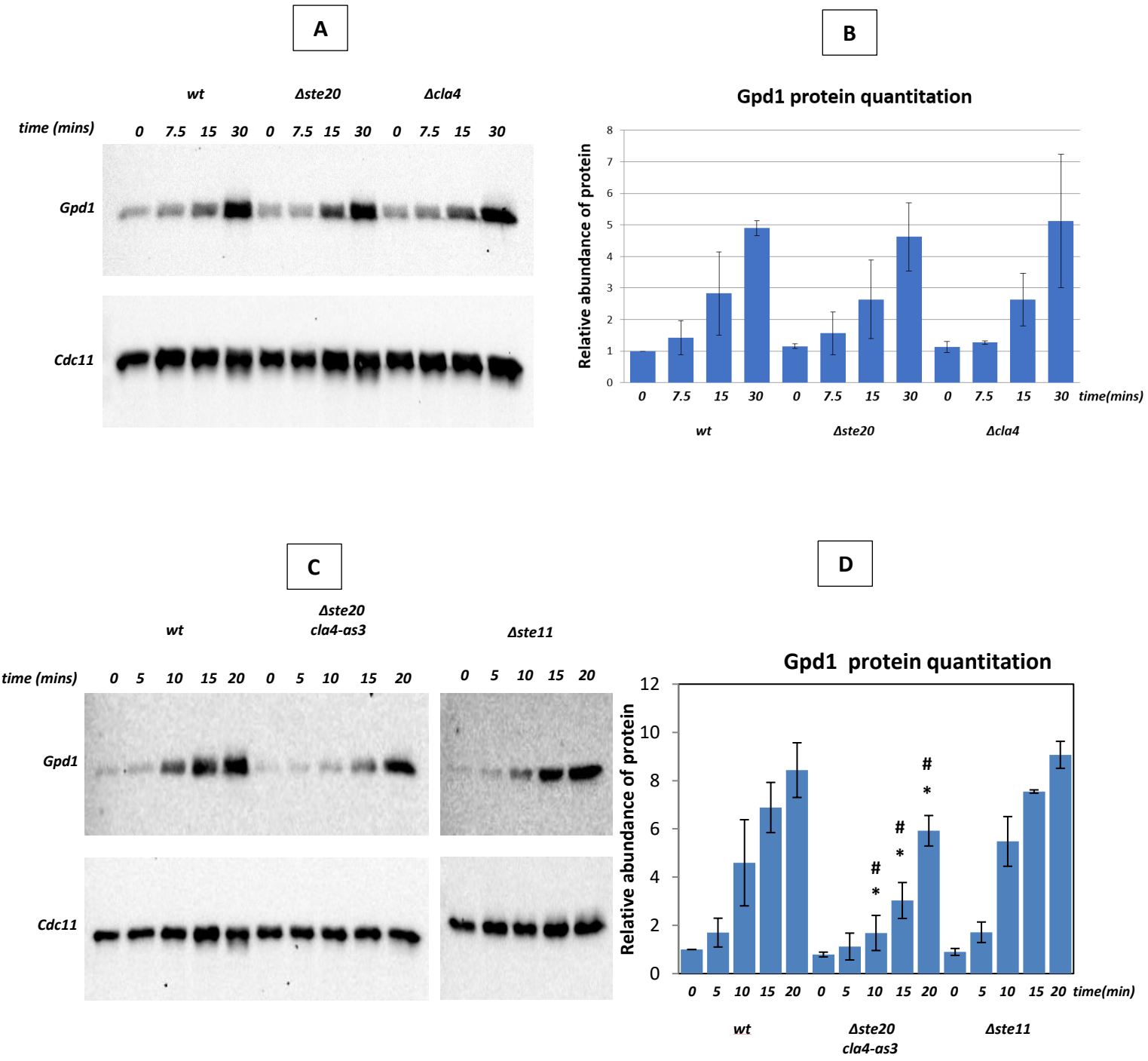


Fig 5.1 Expression level of Gpd1 under hyperosmotic stress. A) Western blot of the kinetic assay of Gpd1 expression level in *Δste20* and *Δcla4* single deletion strains treated with 0.8M NaCl. B) Bar chart of relative abundance of Gpd1 quantitated against Cdc11, a housekeeping protein, in *Δste20* and *Δcla4* cells. C) Western blot showing Gpd1 expression in *Δste20 cla4-as3* double mutant and *Δste11* strains at different time points treated with 0.8M NaCl. D) Bar chart showing Gpd1 levels *Δste20 cla4-as3* double mutant and *Δste11* strains normalised against Cdc11. Results were taken as mean ± SD from three independent experiments. One-way anova statistics tool was used to determine the p-value, p<0.05. *p<0.02 represents significant difference between wt and *Δste20 cla4-as3* while #p<0.02 represents the significant difference between *Δste11* and *Δste20 cla4-as3.*

5.3.2 Phosphorylation status of Gpd1 in *Δste20 cla4-as3* double mutant strain.

Kinases mediate their biochemical effect on their substrates by transferring a phosphate group. PAKs such as Ste20 and Cla4 transfer phosphate groups to serine or threonine residues on their substrates. Studies have shown that activity of Gpd1 is regulated through phosphorylation and here, the effect of Ste20 and Cla4 is investigated in the phosphorylation status of Gpd1. Under hyperosmotic stress condition, Gpd1 expression and activity is upregulated rapidly to switch up the synthesis of glycerol. This forms the basis for the hypothesis behind this study.

Using the Phos-tag SDS PAGE system to detect phosphorylated proteins, fig 5.2 shows the phosphorylation status of Gpd1 in wild type and *Δste20 Δcla4-cla4-as3* double mutant strain under osmotic stress induced with 0.8M NaCl.

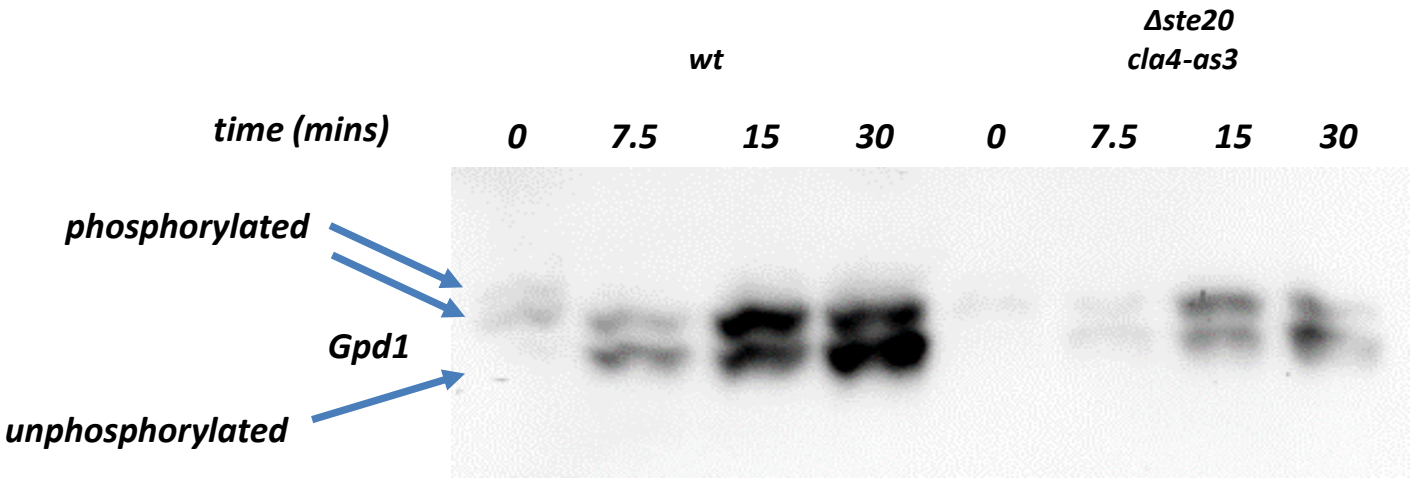


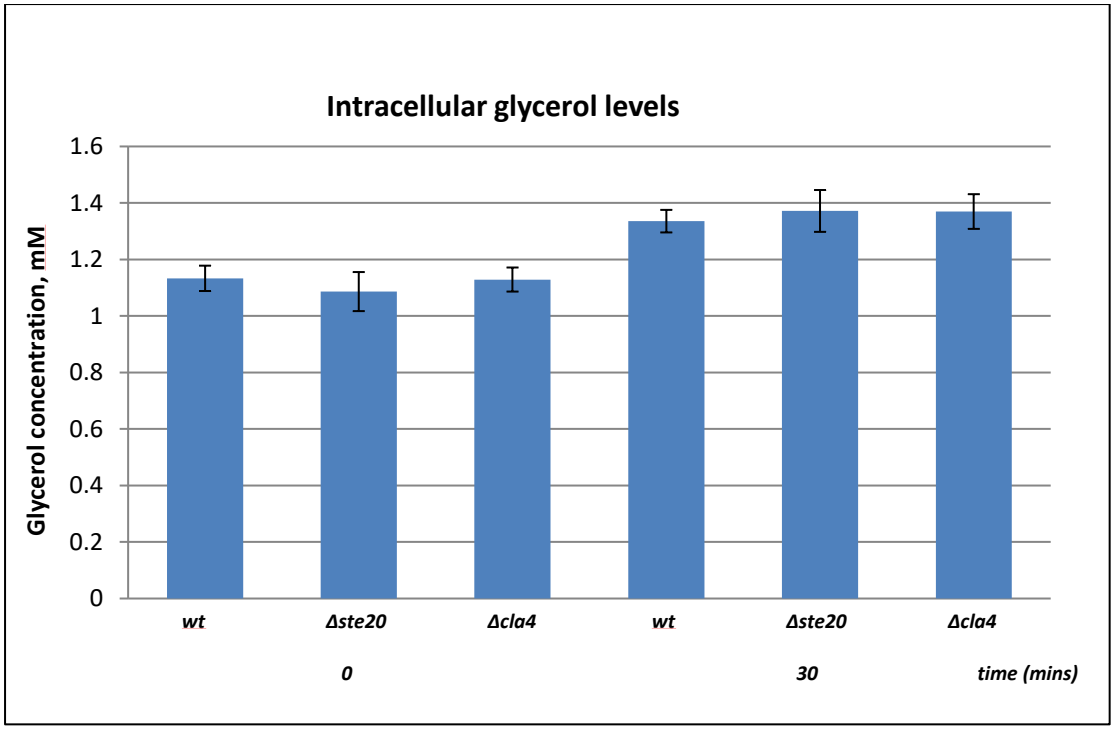
Fig 5.2 Phos-tag SDS-PAGE showing the phosphorylation status of Gpd1 under hyperosmotic stress. Phosphorylation status of Gpd1 protein is investigated in both wild type and a *Δste20 cla4-as3* double mutant strain treated with 0.8M NaCl. Kinetic assay was performed on exponentially growing cells to monitor changes in the level of phosphorylated proteins. Three bands are shown here, top band represents di-phosphorylated proteins, middle band; mono-phosphorylated proteins and the lowest band; unphosphorylated proteins

In the wild type, at 0min, overall protein level seems to be rather low, but it appears more proteins are phosphorylated than they are unphosphorylated as indicated with the two top bands on the membrane indicating di- and mono-phosphorylation respectively. levels of both phosphorylated and unphosphorylated protein began to increase at about 7.5 mins as seen through increase in band intensity which became even more intensified afterwards as seen at time points 15 and 30 mins. Put together, after 0min, overall protein levels went up and there seems to be protein dephosphorylation as the topmost band (bi-phosphorylated protein) is eliminated with a corresponding increase in unphosphorylated proteins. In the double mutant strains, at 0min, overall proteins were lower than those observed in the wild type but there seems to be only phosphorylated proteins. At 7.5mins, overall protein levels remained lower than those in the wild type and this only increased slightly at 15 and 30mins but remained lower than those in the wild type. It appears that similar to what was observed in the wild type, after 0min, there is dephosphorylation of proteins. Put together, level of phosphorylated proteins decreased considerably in *Δste20 cla4-as3* cells. These data showed that loss of Ste20 and Cla4 does not inhibit Gpd1 phosphorylation under osmotic stress but rather this suggests that the observed reduction in phosphorylation status was due to the overall expression of Gpd1.

5.3.3 Levels of intracellular and extracellular glycerol in both *Δste20* and *Δcla4* single mutants and a *Δste20 cla4-as3* double mutant strain.

The direct relationship between increased glycerol synthesis in response to hyperosmotic stress has been well studied. Activated Hog-MAPK pathway that modulates this response has been shown to not just increase glycerol synthesis by increasing the transcription and expression of Gpd1 but to regulate the efflux of glycerol by closing the glycerol transport channel (Lee, et al., 2013). Since Ste20 and Cla4 are involved in the Hog-MAPK pathway during osmoadaption, their direct effect on the synthesis and accumulation of glycerol under this condition was investigated. Since expression of Gpd1 has been shown to be lowered in the *Δste20 Δcla4-cla4-as3* double mutant strain, it is necessary to know whether this also affects glycerol levels. To achieve this, levels of glycerol were measured intracellularly and extracellularly in both *Δste20* and *Δcla4* single mutant and a *Δste20 cla4-as3* double mutant strains.

Fig 5.3 shows both intracellular and extracellular levels of glycerol in *Δste20* and *Δcla4* single mutant strains. Glycerol levels were measured at time points 0, start of osmotic stress induction, and 30. Glycerol levels were represented as mean ± SD and in the wild type, a significant increase in glycerol level was observed after 30mins of stress induction both intracellularly and extracellularly. In both *Δste20* and *Δcla4* strains, levels of intracellular and extracellular glycerol were similar to those of the wild type. This further suggests the overlapping function of Ste20 and Cla4 in the Hog-MAPK pathway.



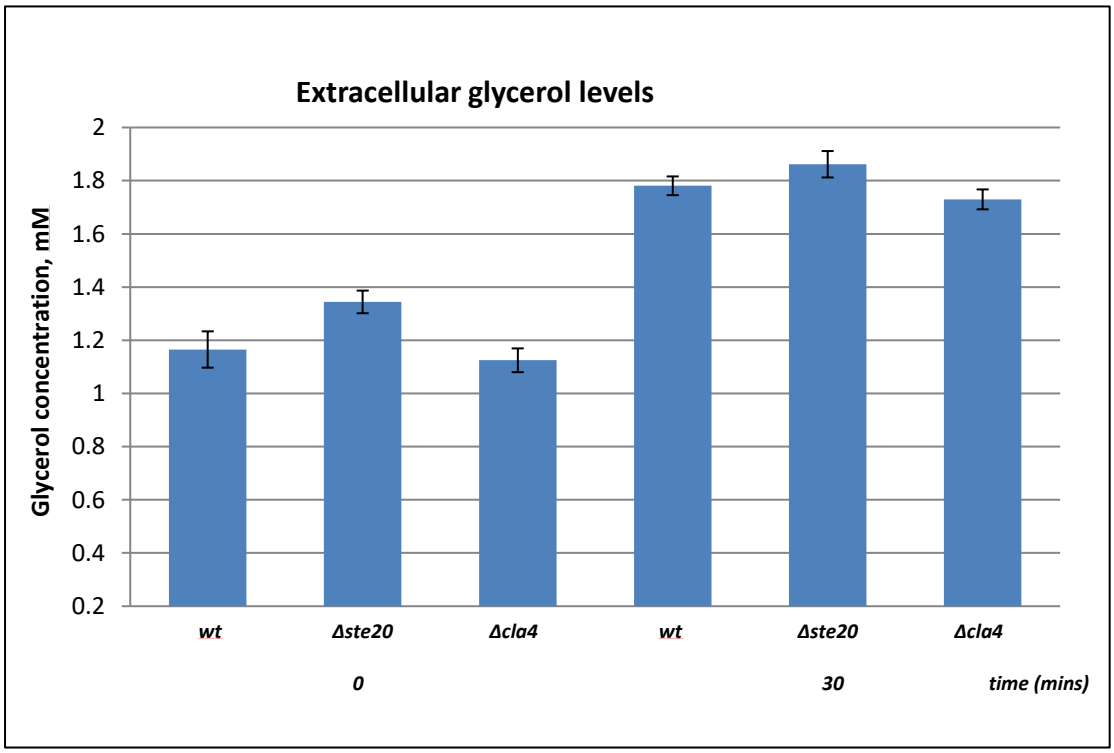


Fig 5.3 Effect of *STE20* and *CLA4* deletion on the synthesis and accumulation of glycerol during hyperosmotic stress. Levels of glycerol were measured intracellularly and extracellularly in single deletion strains of *Δste20 and Δcla4* at the indicated time points. Results are represented as mean ± SD of three independent experiments.

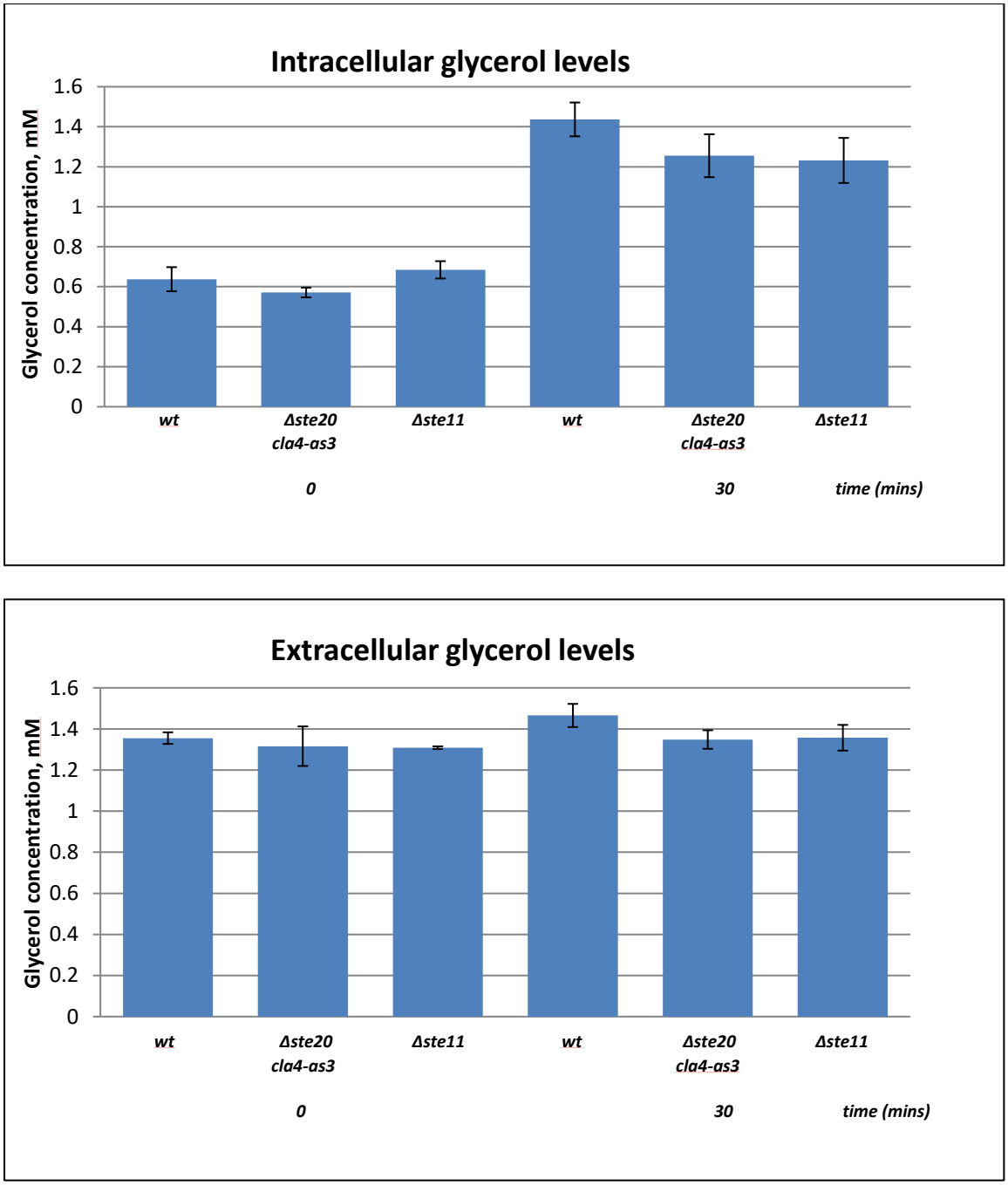


Fig 5.4 Effect of *Δste20 cla4-as3* double mutation on glycerol levels under osmotic stress. Levels of intracellular and extracellular glycerol were measured in double mutant strains and compared to those in *Δste11* strain and wild type strain at indicated time point treated with 0.8M NaCl. Results were represented as mean ± SD taken from three independent experiments.

Levels of glycerol were measured in *Δste20 cla4-as3* double mutant strain and to investigate whether *STE20* and *CLA4* deletion have an effect on synthesis and accumulation of glycerol. Based on the difference in expression level of Gpd1 in *Δste20 cla4-as3* cells and *Δste11* cells,

it was necessary to investigate whether this has an effect on overall glycerol levels under osmotic stress. Fig 5.4 shows that in the wild type, levels of intracellular glycerol increased more than two-fold after 30 mins of osmotic stress induction while extracellular glycerol level was relatively unchanged after 30 mins of stress induction. This observation suggests the relevance of intracellular accumulation of glycerol during osmotic stress. In the *Δste20 cla4-as3* double mutant strain and *Δste11* cells, levels of intracellular glycerol increased considerably and similar to that in the wild type strain while extracellular levels were relatively unchanged and similar to those in the wild type. This result shows that loss of Ste20 and Cla4 has no effect on the intracellular accumulation of glycerol during osmotic stress. Also, extracellular levels were measured since glycerol levels can easily be altered across membrane channels, it was thought that manipulating *STE20* and *CLA4* could affect glycerol levels and measuring the intracellular levels alone could mask this effect. Put together, this data shows that overall glycerol levels are unaffected by the loss of Ste20 and Cla4.

5.4 Discussion

Living cells, including yeast, are constantly being exposed to one type of stress or another. Hyperosmotic stress response happens to be one of the best studied stress cascade in eukaryotes. It has been used as a tool in understanding the molecular mechanism of similar stress conditions in higher eukaryotes, such as mammals, as certain elements found in yeast have homologous pair in these higher eukaryotes - an example is the Hog1-p38 relationship (de Nadal, et al., 2002). The role of the PAKs, Ste20 and Cla4, have been well studied in the regulation of the HOG-MAPK cascade where they upregulate a number of genes, importantly *GPD1*, in combating osmotic stress (Saito & Posas, 2012). As much as is known about their role in accumulation of glycerol in relieving of osmotic stress, their direct effect in the regulation of enzymes involved in glycerol synthesis, particularly Gpd1, under hyperosmotic stress has not been examined.

Studies have shown that under growth in glucose, glycerol is the accumulated and preferred osmolyte during osmotic stress but not in other medium where glucose is not the source of carbon such as in ethanol where trehalose has been reported as the accumulated osmolyte (Babazadeh, et al., 2017). In this study, cells were grown in glucose rich medium and this formed the basis for investigating the regulation of Gpd1. Gpd1 is the predominant isoform of Gpd responsible for glycerol synthesis during hyperosmotic stress and responsible for osmoadaption (Babazadeh, et al., 2014). Here, it was demonstrated the direct effect of Ste20 and Cla4 on the expression of Gpd1 from the minute osmotic stress was induced. In *Δste20* and *Δcla4* single mutants, levels of Gpd1 expression were similar to those in the wild type at the indicated time points. This observation is consistent with reports from several studies of the overlapping function of Ste20 and Cla4 (Bartholomew & Hardy, 2009). When

STE20 and *CLA4* were both conditionally deleted, levels of Gpd1 were significantly reduced compared to those of the wild type after 5 mins of osmotic stress induction. To find out whether the reduced expression of Gpd1 was due to loss of induction of transcription through the Ste20/Cla4-Ste11 branch of the MAPK cascade or due to other functions of Ste20 and Cla4, expression levels of Gpd1 were tested in cells lacking *STE11*. Result showed that levels of Gpd1 in *Δste11* strain was similar to those in the wild type and this probably could be because of the redundancy of the Ssk2/22 proteins, the MAPKKK of the alternative Sln1-HOG-MAPK cascade (see fig 1.7). This suggests that Ste20 and Cla4 not only regulate the transcription of Gpd1 via the Ste20/Cla4-Ste11 branch of MAPK cascade but rather via stability of Gpd1 protein independently of the Ste11-MAPK cascade.

Result of the phosphorylation status in the double mutant strain shows a great reduction in the expression of phosphorylated and unphosphorylated protein compared to the wild type strain. This suggests that Ste20 and Cla4 play a role in stability of Gpd1 resulting in the reduced expression of the phosphorylated protein. One reason behind this is observed from the fact that expression of Gpd1 was similar in both the wild type and *Δste11* and Ste20/Cla4 acts upstream of Ste11 in the MAPK, which further activates a number of kinases that in turn leads to the activation of transcription factors that mediate Gpd1 expression. Results of both expression and phosphorylation status suggest that in addition to what is known of Ste20 and Cla4 in their role on Gpd1 expression, Ste20 and Cla4 could also be playing a role in sustaining the expression of Gpd1 by stabilizing this protein.

Finally, results of the effect of Ste20 and Cla4 on accumulation of glycerol intracellularly and extracellularly shows that deletion of both kinases does not affect the synthesis and accumulation of glycerol. There are some valid explanations to this as studies have shown

that Gpd2 – an isoform to Gpd1 – contributes to the overall Gpd activity and glycerol synthesis in *Δgpd1* osmostressed cells especially when glucose is the main source of carbon (Nevoigt & Stahl, 1997; Nissen, et al., 2000). In addition to this, expression of Gpd1 was not totally absent and the level expressed maybe sufficient enough to contribute to the activity of Gpd and glycerol synthesis.

Chapter 6:

Investigating the effect of Ste20 and Cla4 on the expression and activity of Gpd1 in response to varying glucose levels.

6. Investigating the effect of Ste20 and Cla4 on the expression and activity of Gpd1 in response to varying glucose levels.

6.1 Introduction

Saccharomyces cerevisiae, as well as most organisms, rely on glucose as the most preferred source of carbon for growth and other physiological function. Cells are constantly exposed to varying environmental conditions one of such conditions expose them to limited nutrients such as glucose. Yeast cells respond to the availability of nutrient, such as glucose through several cellular adaptive mechanisms including modification of gene transcription, cellular metabolism and development (Zaman, et al., 2009). Studies have shown that yeast cells perceive and respond to a wide range of glucose concentrations by adapting its growth rate and the expression of a number of growth-rate specific genes (Zaman, et al., 2009). Yeast and certain species of plants and animals, have developed a number of nutrient sensing pathways to overcome and adapt to these periods of nutrient fluctuation in order to carry on with essential cellular processes for survival. One of such important evolutionary response to nutrient limitation in budding yeast is filamentation.

Filamentation is considered an adaptive response by some yeast cells in scavenging for limited nutrient such as glucose, nitrogen (Cullen & Sprague, 2000; Cullen & Sprague Jr., 2012). A number of signal transduction pathways have been reported to regulate filamentation, depending on the nature of limited nutrient (Cullen & Sprague, 2000; Ceccato-Antonini & Sudbery, 2004). A few signal transduction pathways associated with glucose sensing includes the Ras2/cAMP-PKA pathway, Snf1 pathway and the Ste20-Ste11 MAPK pathway (Cullen & Sprague Jr., 2012). Here, the Ste20-Ste11 MAPK pathway of

filamentous growth regulation will be discussed due to the relevance of both proteins in this current study. Upon receiving an adequate stimulus of depleted nutrient, such as glucose or nitrogen, two key transmembrane proteins are activated, Msb2 and Sho1 (fig 1.6), which are found upstream of the filamentous growth response pathway (Cullen, et al., 2004). These transmembrane proteins recruits Cdc42, an essential GTPase of the MAPK cascade, and its associated GEF Cdc24. Cdc42 then recruits and activate the PAKs, Ste20 and Cla4 which then phosphorylate and activate Ste11, a MAPKKK. Ste20/Cla4 and Ste11 are involved in all three MAPK regulated pathways- pheromone response and HOG pathway. How they regulate a particular pathway depends on the nature of stimulus and transmembrane receptor signalling. In the filamentous growth pathway, Ste11 then phosphorylates and activates the MAPKK,Ste7. Ste7 is a protein that also regulates the response to pheromone. However, which pathway it activates depends on whether it interacts with another scaffold protein Ste5 (fig 1.6), which is responsible for the recruitment and orchestrated activation of the Ste11-Ste7-Fus3 cascade in pheromone response. In the absence of Ste5, Ste7 phosphorylates and activates the filamentous growth response specific MAPK, Kss1. Kss1 regulates the activation and expression of the transcription factors Ste12 and Tec1, which controls the regulation of a number of genes.

Other important glucose sensing pathway that also lead to filamentous growth in yeast include the Snf1 and Ras/cAMP-PKA pathway. Studies have shown that some of these glucose-sensing pathways repress a number of genes – when glucose is available – involved in gluconeogenesis, respiration and the metabolism of alternative carbon sources such as sucrose (Gancedo, 1998). When the presence/absence of glucose is sensed, a central glucose-responsive repressor complex is activated/deactivated which controls the

repression of gluconeogenic genes, respiratory gene and those involved in the utilization of galactose, maltose and sucrose. This occurs via the Snf1 kinase pathway (Carlson, 1999). The TORC2-Gad8 is activated by the cAMP-PKA pathway when cells growing on nonfermentable carbon sources sense the presence of glucose via the Gpr1/Gpa2 G-protein coupled receptor system (Rolland, et al., 2002). This leads to the increase in cAMP levels regulated by the adenylate cyclase Cyr1; whose activity in yeast is controlled by the Ras proteins. PKA is then activated by cAMP regulating a number of transcription genes involved in sugar metabolism, stress resistance and growth such as Msn2 and Msn4, anti-stress response transcription factors, (Thevelein & de Winde, 1999) and TORC2; a stress response gene (Cohen, et al., 2014).

6.2 Aim

- To investigate the effect of Ste20 and Cla4 on the expression of Gpd1 in response to varying glucose levels.
- To understand the role of Ste20 and Cla4 in the phosphorylation and activity of Gpd1 in response to varying glucose levels.
- To investigate whether the deletion of Ste20 and Cla4 play any significant role in the synthesis of glycerol in response to varying glucose levels.

6.3 Results

6.3.1 Expression of Gpd1 in single deletion strain of *STE20* and *CLA4* and a *Δste20 cla4-as3* double mutant strain in response to varying glucose levels.

Here, the role of Ste20 and Cla4 was investigated in the expression of Gpd1 in response to glucose. The rationale behind this test came from the knowledge of the role of Ste20/Cla4 in filamentation and because from the outcome of the experiments above, Ste20 has been shown to interact with Gpd1 and exert a crucial role on its expression. Hence, it was necessary to test whether Ste20-Gpd1 link has any effect on filamentation. To achieve this, cells were grown under four conditions. The first condition requires cells grown in a medium lacking glucose, YP, in the second condition, cells were grown in a glucose-rich medium, YPD, in the third condition, cells in the first and second condition were grown on solid medium, agar plates and finally, for the fourth condition, cells from the first and second condition were grown in liquid medium as shown in fig 6.1. Under experimental conditions, filamentation is triggered in budding yeast when cells are grown on solid medium lacking glucose. For all four conditions, cells were grown overnight to an OD_{600} 0.5 in YPD, washed and grown to the same density, by means of serial dilution with water, on agar plates and liquid medium with and without glucose. Cells were incubated for about 12 hours at 30^{o}C.

Fig 6.1a-b shows western blot of Gpd1 expression and Cdc11 in all test conditions and bar chart showing relative abundance of Gpd1 obtained from normalisation with Cdc11. Levels of Gpd1 in medium lacking glucose on agar plates were relatively similar to those in medium containing normal glucose level while in the liquid culture, levels of Gpd1 were considerably reduced in medium lacking glucose compared to those in medium containing normal glucose level. This result suggests that regulation of Gpd1 levels were not specific under

conditions that trigger filamentation but rather its level seem to be regulated in response to normal level of glucose in liquid medium.

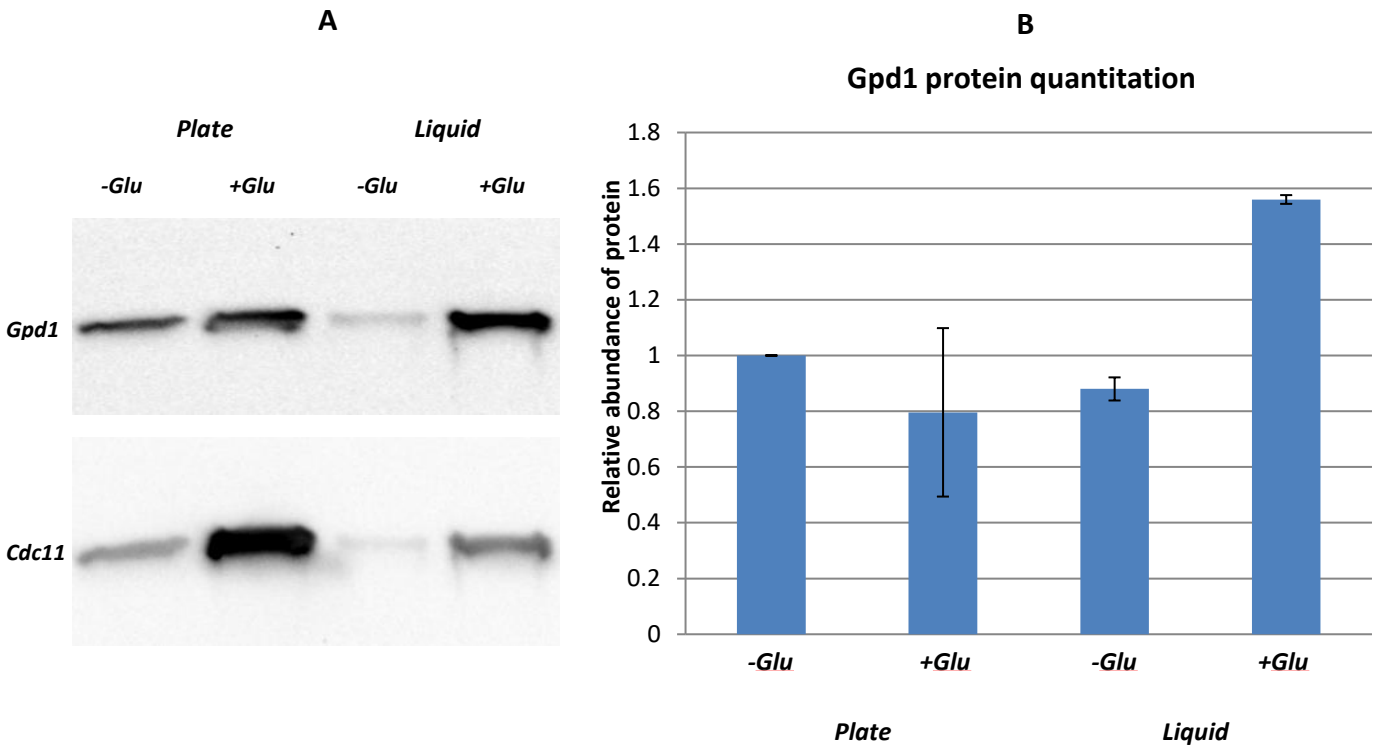


Fig 6.1 Gpd1 expression during filamentation. A) Western blot of Gpd1 expression and Cdc11 on agar plates lacking glucose (which triggers filamentation) and containing normal glucose concentration and in liquid medium lacking glucose and containing normal glucose concentration. Same cell density was achieved for medium lacking glucose and medium with normal glucose level by diluting overnight cultures washed with water. B) Bar chart showing relative abundance of Gpd1. Gpd1 levels in all test conditions were normalised against Cdc11. Results are represented as mean ± SD from three independent experiments.

Since Gpd1 level increased considerably in liquid medium containing normal glucose level, the next part of this study will be to characterise the link between Gpd1 expression and glucose levels. To achieve this, wild type cells grown in YPD medium overnight were transferred to a fresh medium containing normal glucose level and grown for 3 hours. 2% glucose was added to exponentially grown cells and Gpd1 levels were measured at different time points as shown in fig 6.2. Levels of Gpd1 considerably increased immediately after 2%

glucose was added, between 0 to 7.5mins. This increase was observed further at 15 and 30mins. Overall, levels of Gpd1 increased by at least two-fold after 30mins of adding 2% glucose. This result shows that *GPD1* is upregulated in response to varying glucose level.

Since Gpd1 expression in response to varying glucose level has been established in the wild type, it was therefore necessary to investigate whether Ste20 and Cla4 exert any role on Gpd1 expression in response to the addition of glucose. To analyse this, Gpd1 expression was investigated in both *Δste20* and *Δcla4* single mutant strains (fig 6.2) and *Δste20 cla4-as3* double mutant strain (fig 6.3) in response to 2% glucose.

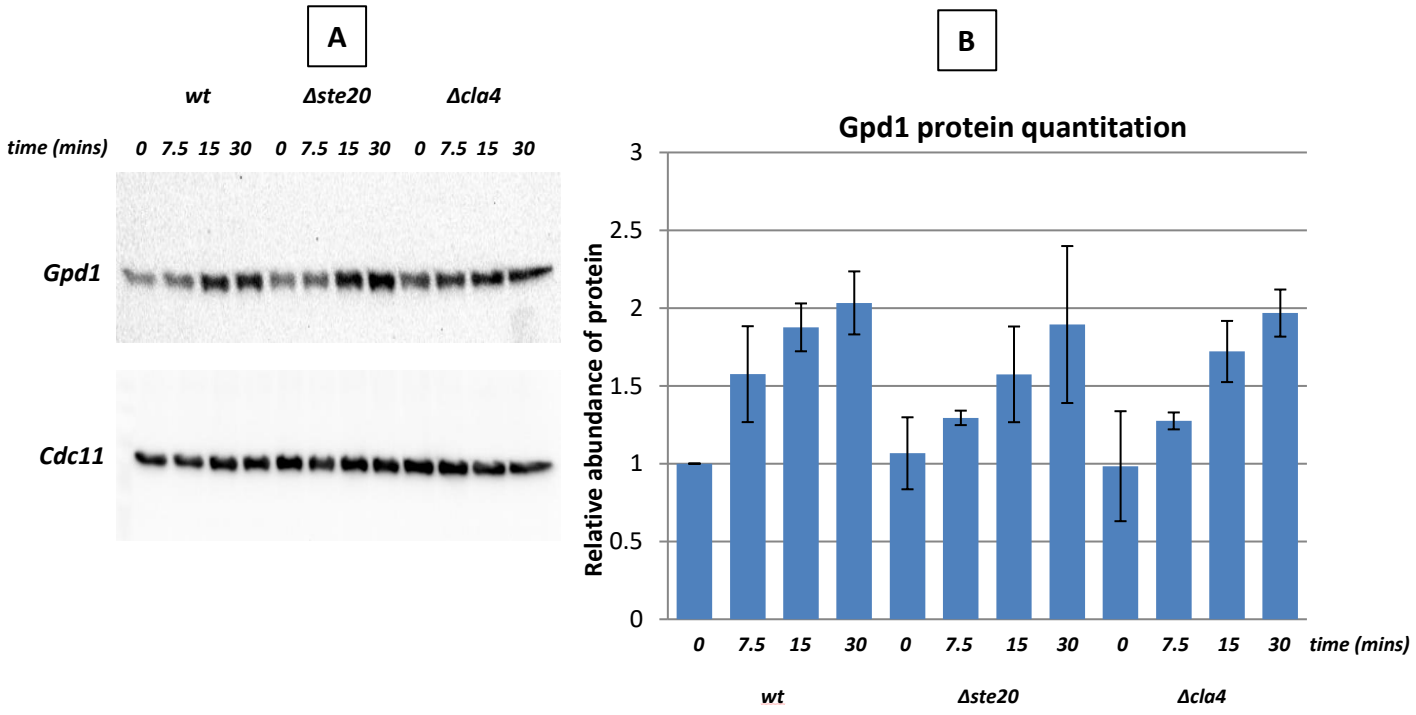


Fig 6.2 Gpd1 expression in *Δste20* and *Δcla4* cells in response to 2% glucose. A) Western blot of Gpd1 and Cdc11 expression at different time points. B) Bar chart of relative abundance of Gpd1 levels obtained from normalisation with Cdc11 as a ratio of Gpd1:Cdc11 in wt, *Δste20* and *Δcla4* cells. Results are represented as mean ± SD from three independent experiments.

Fig 6.2a shows western blot of Gpd1 expression measured against Cdc11, a housekeeping protein. fig 6.2b shows bar chart of relative abundance of Gpd1 obtained as a ratio of Gpd1 and Cdc11 for each time point. In the wild type, levels of Gpd1 increased by two-folds after 30mins of glucose addition. In the *Δste20* and *Δcla4* single mutant strains, levels of Gpd1 also increased by about two-folds and were similar to those of the wild type strain. This suggests that deletion of either Ste20 and Cla4 have no effect on Gpd1 expression.

Since Ste20 and Cla4 have overlapping functions over a wide range of cellular process in yeast, it was necessary to test whether a double mutation of both would have any effect. Fig 6.3a shows western blot of Gpd1 and Cdc11 expression in wild type and *Δste20 Δcla4-cla4-as3* double mutant strain at different time points. Levels of Gpd1 were normalised against levels of Cdc11 by obtaining the ratio of Gpd1:Cdc11 expression at each time points. This gives the relative abundance of Gpd1 level as shown on the bar chart in fig 6.3b. Levels of Gpd1 in the wild type slightly increased from when glucose was added to 15 mins by less than two-fold but a significant increase of more than two-fold was noticed after 15 mins up till 30 mins which remained relatively the same at 45mins. In the *Δste20 cla4-as3* double mutant strain, levels of Gpd1 slightly increased from when glucose was added to 15 mins similar to what was obtained in the wild type. However, after 15mins, Gpd1 levels remained relatively the same and when compared to those in the wild type, after 15mins, levels of Gpd1 were significantly reduced by more than two-folds. Also, it was important to test whether the observed decrease was due to loss of induction of transcription through the Ste20/Cla4-Ste11 branch of the MAPK cascade or due to other unknown functions of Ste20/Cla4. In the *Δste11* strain (fig 6.3), levels of Gpd1 were similar to those in the wild

type at all indicated time points. When compared to levels in the *Δste20 cla4-as3* double mutant strains, levels in the *Δste11* strain significantly increased by two-folds after 15mins of glucose addition as observed in the wild type. This suggests that the observed decrease in Gpd1 level observed was independent of the Ste20/Cla4-Ste11 branch of the MAPK cascade but rather Ste20/Cla4 have a direct role in the regulation of Gpd1.

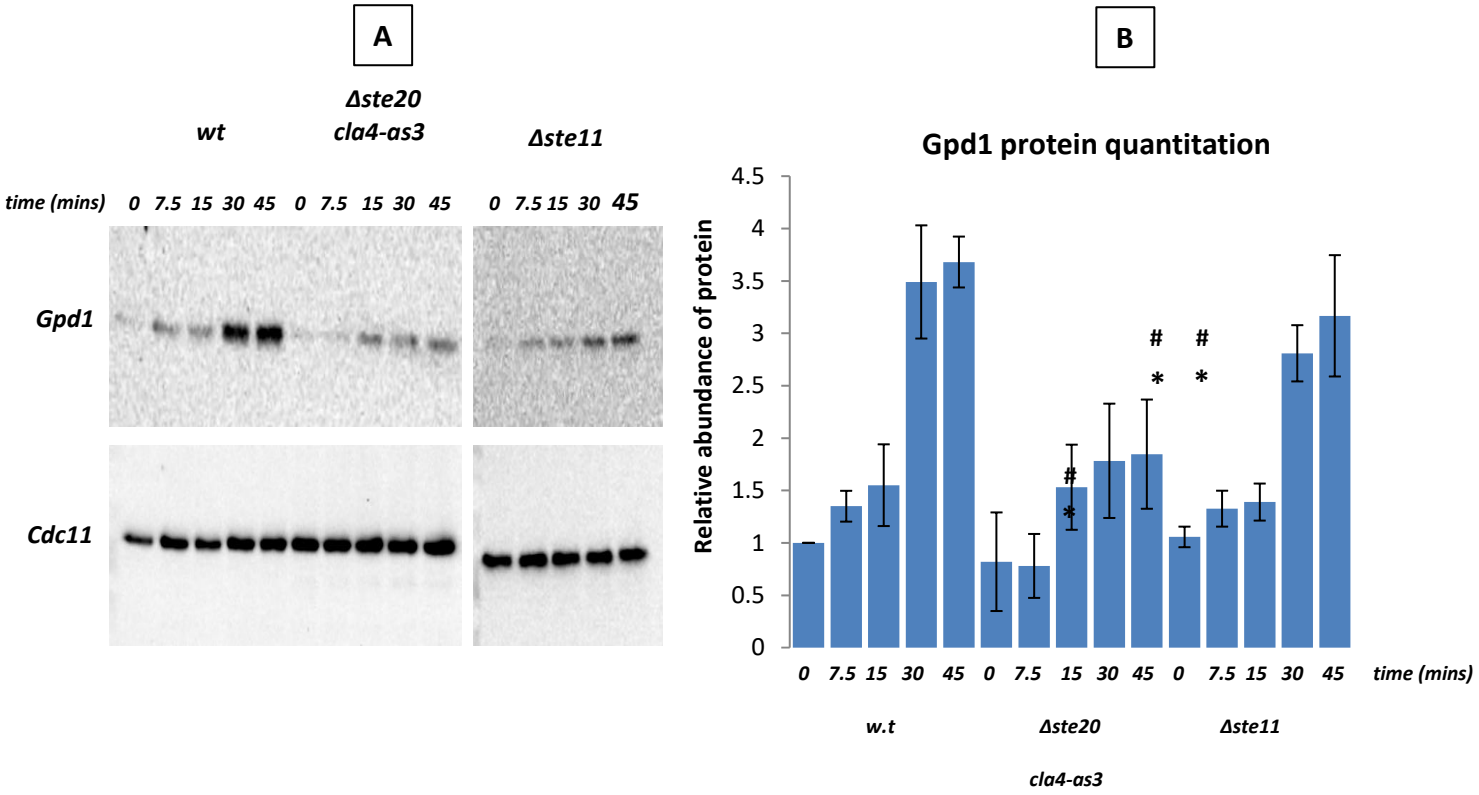


Fig 6.3 Effect of *Δste20 cla4-as3* double mutation and *Δste11* single deletion on expression level of Gpd1 in response to 2% glucose. A) Western blot of Gpd1 and Cdc11 expression at different time points. B) Bar chart of relative abundance of Gpd1 normalised against Cdc11. Gpd1:Cdc11 ratio was obtained to normalise Gpd1 levels for each time point. P-values, p<0.05, was obtained using the one-way anova statistics tool. *p<0.05 indicates significant difference between wild type and *Δste20 cla4-as3* while #p<0.05 indicate significant difference between *Δste11* and *Δste20 cla4-as3*. Results are represented as mean ± SD from three independent experiments.

Based on the evidence of the pattern of decrease of Gpd1 protein level in the double mutant strain (also in the test conditions, section 4 and 5), it was observed that Gpd1 levels

decreased after overnight starvation (glucose depletion) but remained rather unchanged after switching to a fresh medium with normal glucose concentration (section 4.3) for a few hours. Hence, Gpd1 levels seem to decrease slowly after long exposure with normal glucose concentration. This slow decrease of Gpd1 protein levels could be explained by a reduction in transcription and/or transcription. To analyse this, a cycloheximide chase assay was performed to determine the half-life of Gpd1. Cycloheximide is a potent antibiotic, widely used for the inhibition of protein synthesis by preventing translational elongation (Bahram, et al., 2000; Zhou, 2004; Oh, et al., 2017).

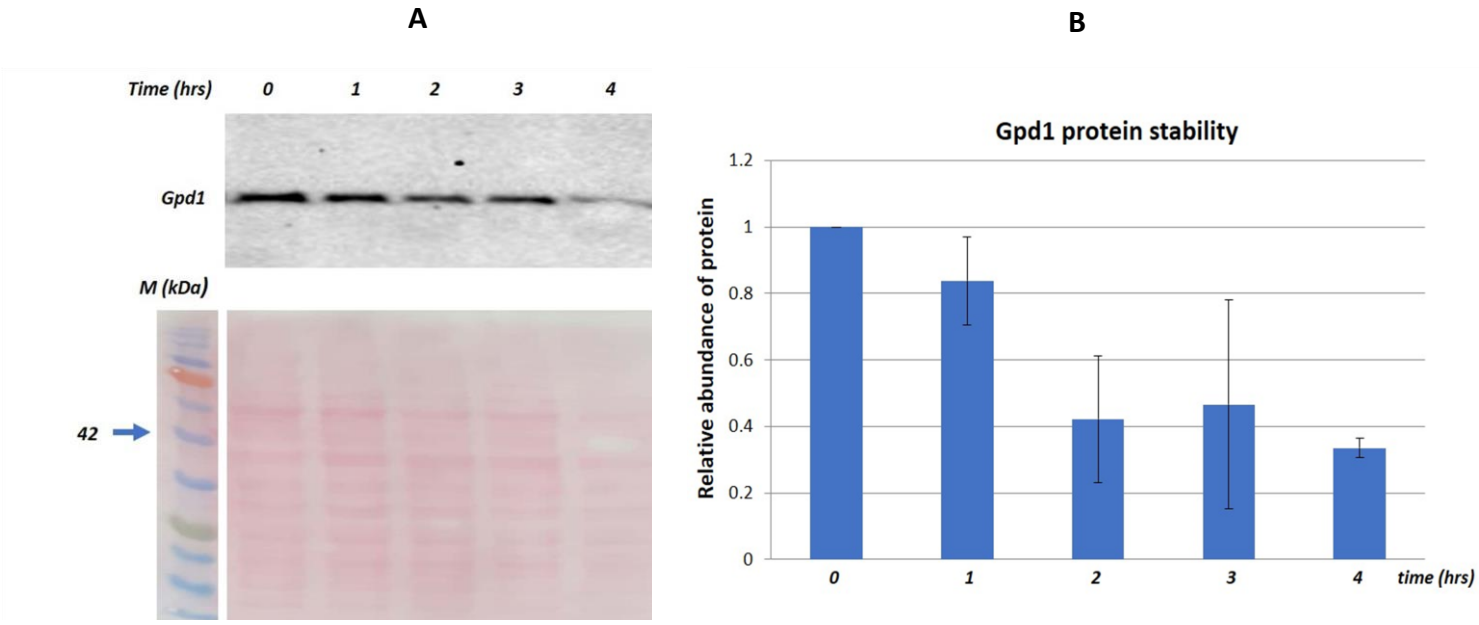


Fig 6.4. Determination of Gpd1 half-life. A) Western blot of Gpd1 protein expression and the ponceau S stain. Exponentially grown cells were treated with 100µg/ml cycloheximide and samples were collected for protein expression at indicated time points. 12% SDS-PAGE was used to resolve proteins which were transferred to a nitrocellulose membrane and stained with ponceau S to ensure same amount of protein sample was loaded in each lane. B) Bar chart showing relative abundance of Gpd1 protein at each time point. Results are shown as mean ± SD from three independent experiments.

In this study, cells were grown overnight, transferred to a fresh medium containing normal glucose concentration and grown to an exponential phase for about 3 hours. Afterwards,

cells were treated with cycloheximide for up to 4 hours. As shown in fig 6.4, after 2 hours, levels of Gpd1 reduced to half its relative quantity. Gpd1 protein level reduced further but only slightly at 4 hours. In a proteome-wide study, Gpd1 half-life was published as 25 hours but this study shows a much shorter time of about 2 hours. This shows that Gpd1 levels remain the same after 4 hours of growth in medium with normal glucose concentration but dropped after the same time of protein biosynthesis inhibition with cycloheximide. It therefore seems likely that transcription/translation is not affected immediately after reducing glucose concentrations

6.3.2 Phosphorylation status of Gpd1 in *Δste20 cla4-as3* double mutant strain.

Since Gpd1 has been shown to be a phosphoprotein and the conditional mutation of both kinases shown to have an effect on its expression level in response to addition of glucose, it was important to test the effect of the conditional mutation of Ste20 and Cla4 on the phosphorylation status of Gpd1.

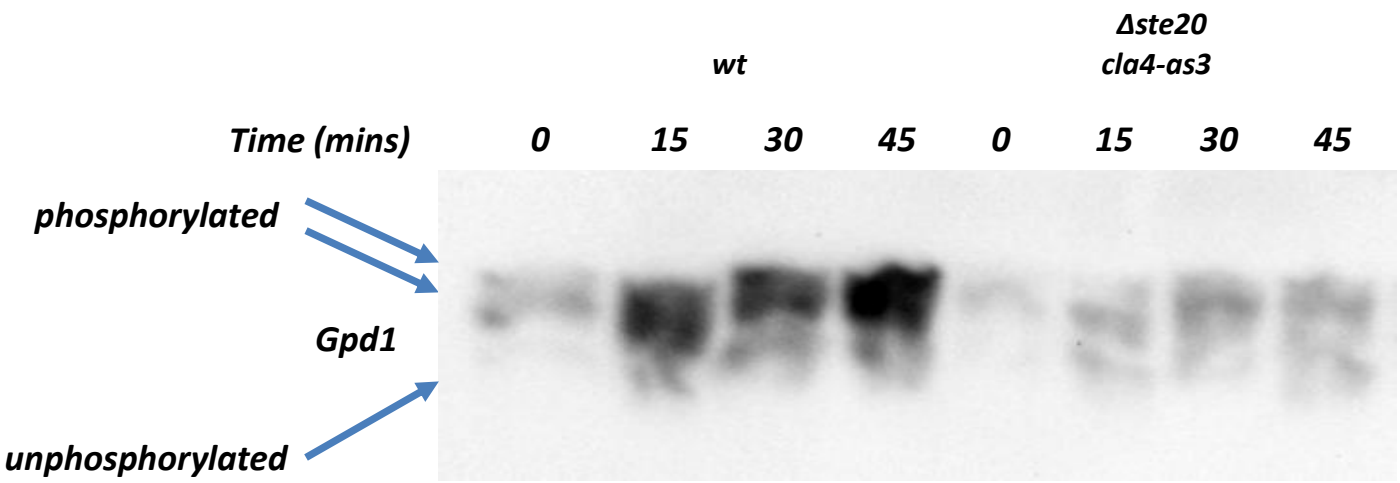


Fig 6.5 phosphorylation status of Gpd1 in response to 2% glucose. Expression levels of phosphorylated and unphosphorylated protein in wild type and *Δste20 cla4-as3* double mutant strains. Cells grown overnight were transferred to fresh YPD, treated with 10μM 1NM-PP1 and grown for 3hours to an exponential phase. 2% glucose was added to cells and immediately protein samples was extracted from cells indicated as time point 0. Protein samples was then taken for each indicated time point.

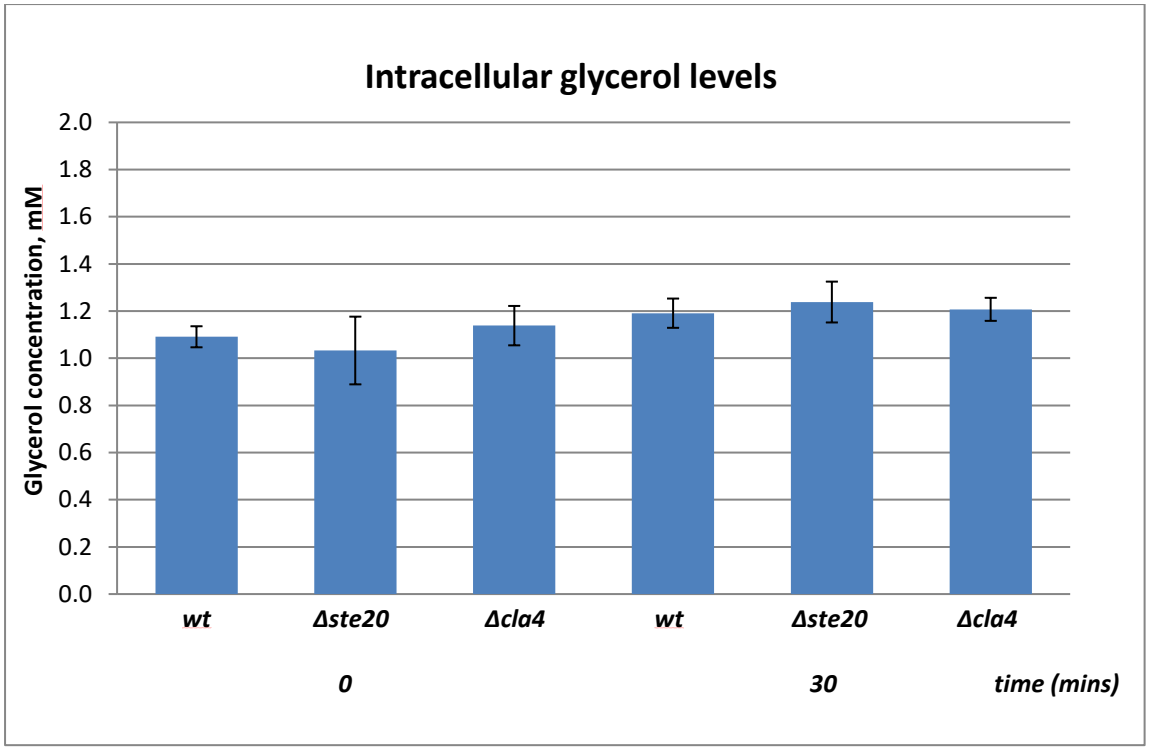
Cells were grown overnight and transferred to fresh YPD medium. To inhibit the kinase activity of the double mutant strain carrying the *cla4-as3* allele plasmid, cells were treated with the kinase inhibitor 1NM-PP1 and grown to an exponential phase and were then treated with 2% glucose (v/v). Cell samples were taken at different time points as indicated in fig 6.4 and proteins were extracted and resolved using phos-tag SDS PAGE. Fig 6.5 shows that in the wild type, expression level of phosphorylated Gpd1 greatly increased after 15 mins of 2% glucose addition and this level was relative constant after another 15 mins (time point 30) while a slight increase was observed at 45 mins. Levels of unphosphorylated

proteins (lowest band) were also slightly increased from time point 0 to 45. It is also important to mention here that at least two phosphorylation states of Gpd1 were observed, the top-most band being doubly phosphorylated and the next being mono-phosphorylated. The ratio of expressed levels of phosphorylated to unphosphorylated proteins greatly increases as well from time point 0 – 45. In the *Δste20 cla4-as3* double mutant strain, levels of phosphorylated and unphosphorylated proteins only slightly increased after 15mins of glucose addition. This level remained relatively unchanged 30mins later (time point 45). Ratio of phosphorylated and unphosphorylated were relatively unchanged across all time points. When compared to those in the wild type, levels of phosphorylation were remarkably reduced after 15mins and even remained relatively unchanged at 30 and 45 mins. This shows that both Ste20 and Cla4 affect the phosphorylation status of Gpd1 in response to glucose.

6.3.3 Levels of intracellular and extracellular glycerol in both *Δste20 and Δcla4* single deletion and *Δste20 cla4-as3* double mutant strains.

It has been shown in this study that Ste20 and Cla4 are required for glucose-induced increase in Gpd1 protein levels. It is therefore necessary to test whether this has an effect on glycerol levels under the same condition. Here, levels of both intracellular and extracellular glycerol were measured in both single mutant strains of *STE20* and *CLA4* and the double mutant strains in response to 2% glucose. Cells grown overnight were transferred to fresh YPD medium and grown to an exponential phase before 2% glucose was added. Fig 6.6 shows that intracellular and extracellular glycerol levels in the wild type were relatively unchanged after 30 mins of glucose addition. In the Δste20 and Δcla4 single deletion strains, intracellular and extracellular glycerol levels were unchanged after 30mins of glucose addition and were similar to those in the wild type.

Fig 6.7 shows the intracellular and extracellular glycerol levels in the *Δste20 cla4-as3* double mutant strains. In the wild type, levels of intracellular and extracellular glycerol were unchanged after 30mins of 2% glucose addition while in the double mutant strain, intracellular and extracellular glycerol levels were unchanged after 30mins and similar to those in the wild type at the indicated time points. This shows that although Ste20 and Cla4 regulate the expression level of Gpd1 protein in response to glucose, they do not affect the level of glycerol both intracellularly and extracellularly.



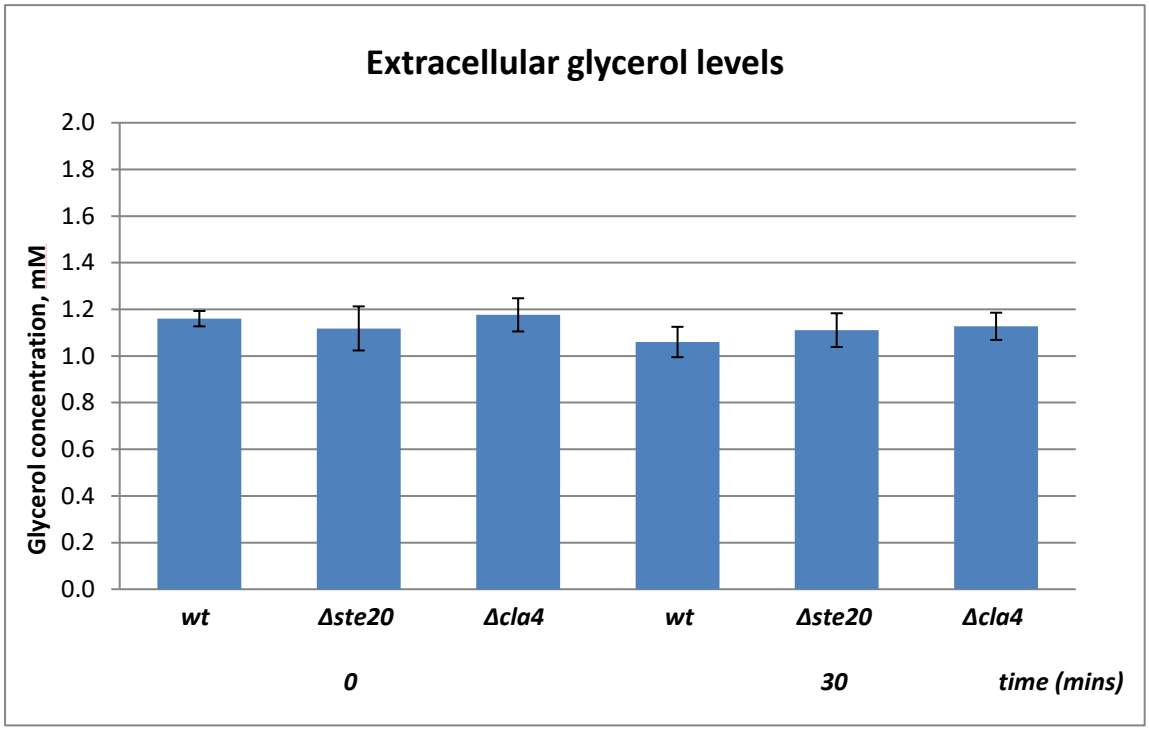
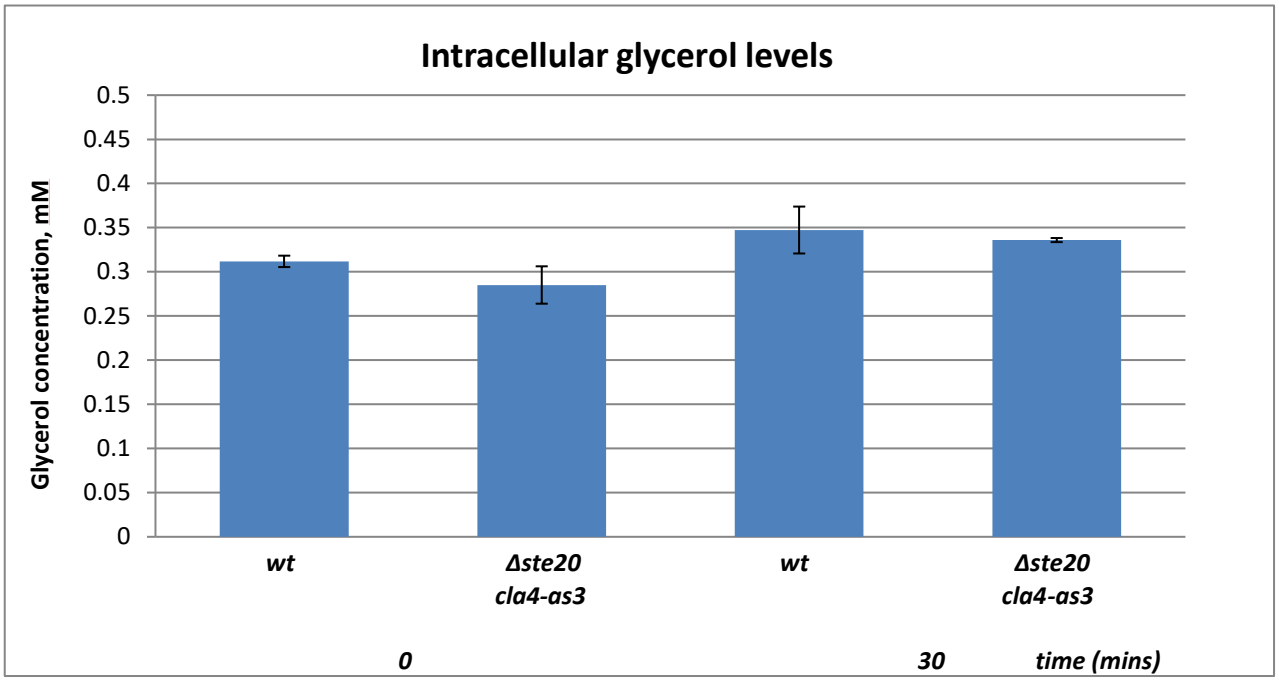


Fig 6.6. Levels of intracellular and extracellular glycerol in response to 2% glucose. Levels of glycerol were measured in *Δste20 and Δcla4* single deletion strains and compared with the wild type levels at indicated time points. Results are represented as mean ± SD from three independent experiments.



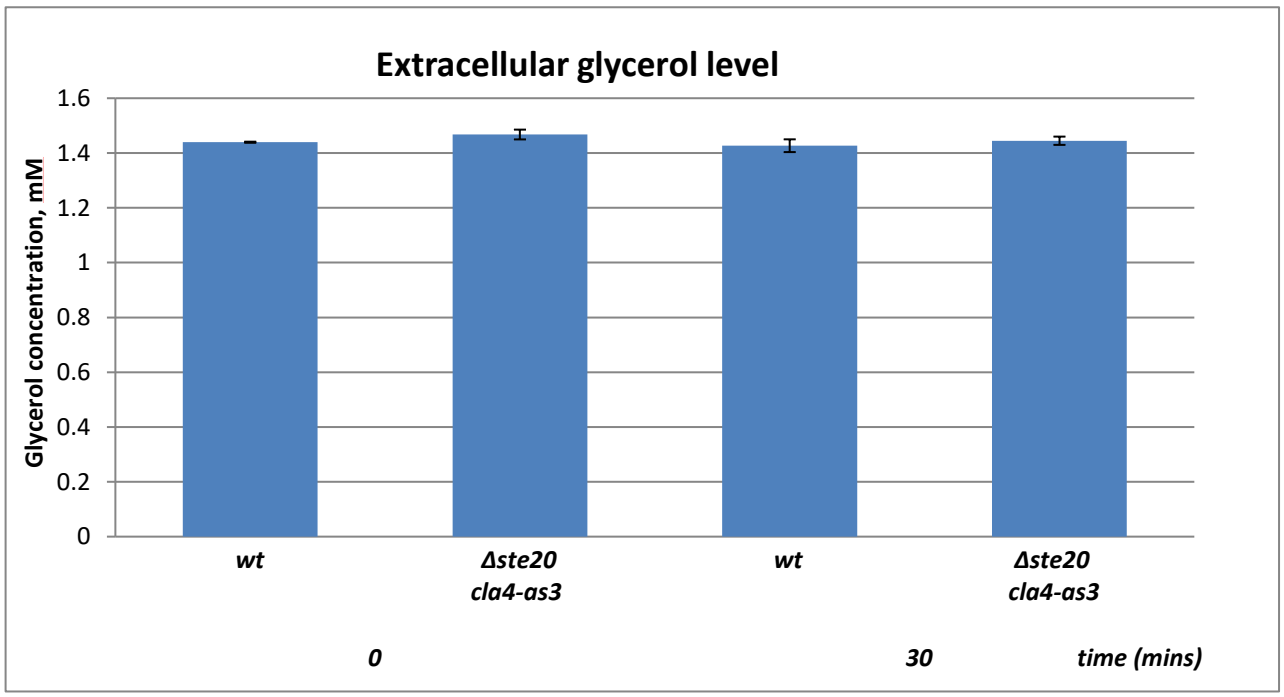


Fig 6.7. Intracellular and extracellular glycerol levels in response to 2% glucose. Levels of glycerol were measured in *Δste20 cla4-as3* double mutant strain and compared to those in the wild type. Results were represented as mean ± SD from three independent experiments.

6.4 Discussion

Cells sense, respond and adapt to nutrient availability by modifying a range of cellular processes such as modifying transcriptional, metabolic and developmental events to adequately adjust to the specific nutrient in question (Zaman, et al., 2009). Addition of glucose to growing cells have been shown to cause a rapid change in the pattern of the overall transcriptional state of the genome as well as protein phosphorylation (Wang, et al., 2004). To understand whether Ste20 and Cla4 have a regulatory effect on protein regulation of Gpd1, it was important to test whether there is link between Ste20/Cla4 and Gpd1 in the regulation of filamentation – a process well characterised by nutrient availability such as glucose and which Ste20 seems to play an essential role while Cla4 seems only important. (Cullen & Sprague Jr., 2012). Here, levels of Gpd1 were shown to be relatively unchanged under conditions which triggers filamentation, cells grown on agar plates lacking glucose, when compared to those with the presence of glucose. However, Gpd1 levels were significantly reduced, about two-folds, in liquid culture with no glucose when compared to those with glucose. This shows that Gpd1 levels were relatively unchanged under conditions that trigger filamentation (on agar plates) but rather increased in response to varying glucose in liquid medium. This suggests that *GPD1* expression might correlate with glucose levels.

This observed increase in Gpd1 level in liquid medium with glucose compared with growth in liquid medium lacking glucose correlates with the increase in Gpd1 expression when cells were grown under optimal growth condition in YPD (section 4.3). It seems Gpd1 levels are lowered when cells are starved overnight due to the depletion of glucose, which is similar to levels in growth in liquid medium lacking glucose. After starved cells were transferred to a

fresh YPD medium, containing normal glucose level required for optimal growth of budding yeast under experimental condition, levels of Gpd1 increased by about two-fold after 30mins (fig 4.2) which is similar to Gpd1 level in the liquid medium with glucose.

In this study, the role of Ste20 and Cla4 was investigated in the regulation of Gpd1 in response to glucose. In the wild type, levels of Gpd1 increased considerably by more than two-fold after 30mins of glucose addition. This observed increase suggests there is a link between increase in glucose concentration and Gpd1 levels. Based on the observation in the wild type, it was necessary to investigate whether there is any link between *STE20* and *CLA4* and glucose-induced Gpd1 increased expression. Single deletion of *STE20* and *CLA4* showed a similar expression level of Gpd1 to those of the wild type strain. This suggests that Ste20 and Cla4 may have no effect on the regulation of Gpd1 protein levels in response to 2% glucose. Levels of Gpd1 protein were significantly decreased in the *Δste20 cla4-as3* double mutant strain when compared to those in wild type which shows that Ste20 and Cla4 play an essential role in regulation of Gpd1 protein level. Since levels of Gpd1 were significantly reduced only when both kinases were mutated and not when either was present in the wild form, this suggests that Ste20 and Cla4 have overlapping functions in the regulation of *GPD1* expression in response to varying glucose levels. It has been reported of the possibility of both Ste20 and Cla4 to function redundantly over a wide range of cellular process (Cvrckova, et al., 1995). However, since it has been established that Ste20/Cla4, MAPKKKK, activate Ste11, a MAPKKK, in the regulation of a number of genes via the MAPK pathway (Rensing & Ruoff, 2009), it was necessary to test whether the decrease in Gpd1 protein level was due to loss of induction of transcription via the Ste20/Cla4-Ste11 cascade or maybe by an unknown Ste20/Cla4 role. Gpd1 levels in the *Δste11* single mutant strains were similar to those of the

wild type and a significant increase was observed when compared to those in *Δste20 Δcla4-cla4-as3* double mutant strain. This shows that the observed decrease in Gpd1 protein level is not due to loss of induction of transcription via the Ste20/Cla4-Ste11 cascade but rather by an unknown role of Ste20/Cla4.

Also, the phosphorylation status of Gpd1, a multi-phosphoprotein (Oliveira, et al., 2012), was investigated. Protein phosphorylation has been reported to be of central importance in yeast central carbon metabolism (Oliveira & Sauer, 2012; Chen & Nielsen, 2016). In this study, phosphorylation status of Gpd1 was investigated whether Ste20 and Cla4 have an effect on it using phos-tag SDS-PAGE system to detect phosphorylated proteins. In the wild type, after 15mins of glucose addition levels of phosphorylated protein greatly increased and was sustained up till 45mins. Blot images showed Gpd1 was phosphorylated singly and doubly and this has been reported of Gpd1 when grown in glucose (Oliveira, et al., 2012). Levels of unphosphorylated proteins also increased in the same manner although the ratio of phosphorylated to unphosphorylated proteins increased greatly. In the *Δste20 Δcla4-cla4-as3* double mutant strains, levels of phosphorylated proteins only slightly increased and when compared to those of the wild type, phosphorylation was greatly reduced. This data clearly indicates that the reduction in phosphorylation observed is as a result of the low level of overall protein expression. Put together the result of Gpd1 expression level and phosphorylation status, both suggest that in addition to what is known of Ste20 and Cla4 and their role in *GPD1* expression, they could also be involved in the stability of Gpd1 protein.

Yeast cell adjusts to the presence of glucose and or modify its growth rate in the presence of glucose by accumulating glycerol which is achieved by upregulating its synthesis (Nevoigt &

Stahl, 1997). Since it has been shown that levels of Gpd1 and its phosphorylation status were significantly reduced in the *Δste20 Δcla4-cla4-as3* double mutant strain, it was important to test whether this also has an effect on glycerol levels since Gpd1 is the most active form of *GPD* involved in the synthesis of glycerol in response to growth in glucose as the sole source of carbon (Nevoigt & Stahl, 1997; Oliveira, et al., 2012). Levels of glycerol were tested for both within the cell, intracellular and outside of the cell, extracellular. The rationale behind this is because several studies have accounted for the possibility of an efflux of glycerol freely by both simple diffusion and via a dedicated Fps1 glycerol transport channel under osmostress-free conditions (Luyten , et al., 1995; Nevoigt & Stahl, 1997; Mager & Siderius, 2002). In the wild type, levels of both intracellular and extracellular glycerol were unchanged after 30mins of glucose addition and in the *Δste20* and *Δcla4* single deletion strains, levels were unchanged after 30mins and were similar to those in the wild type. Since single deletion of either Ste20 and Cla4 had no effect on Gpd1 expression, this shows that deletion of either has no effect on either intracellular and extracellular glycerol levels. In the *Δste20 Δcla4-cla4-as3* double mutant strain, levels of Gpd1 expression were significantly reduced but intracellular and extracellular glycerol levels were relatively unchanged and were similar to those in the wild type. This shows that although in the absence of both Ste20 and Cla4, Gpd1 expression levels are reduced but this has no effect on accumulation of glycerol. One explanation to this effect is that the amount of Gpd1 expressed in the double mutant strain is sufficient enough to initiate synthesis of glycerol up to levels observed in the wild type as it has been reported that under conditions of total absence of Gpd1 such as in *Δgpd1,* is the synthesis of glycerol significantly reduced (Nevoigt & Stahl, 1997; Oliveira, et al., 2012). Another explanation could be the result of an

alternative pathway towards glycerol synthesis with one reported in yeast via dihydroxyacetone pathway (Norbeck & Blomberg, 1997).

Chapter 7:
Discussion

7 Discussion

7.1 Protein-protein interactions between Ste20/Cla4 and enzymes of glucose metabolism

Understanding many processes within the cell is achievable when a good knowledge of proteins involved in such cellular processes are known. Cell biologists, biochemists and other molecular scientist struggle to identify and understand the molecular and functional properties of proteins. One key way in deciphering the complex characteristics of individual proteins is to investigate how they interact as it appears they rarely act alone but rather exist as a complex with other molecules (De Las Rivas & Fontanillo, 2010). Understanding protein-protein interactions (PPI) provides an insight to a wide range of biological processes including cell division, metabolism, cell signalling and developmental control (Braun & Gingras, 2012). PPIs can be classified in several ways based on structural and functional characteristics (Nooren & Thornton, 2003). They may be transient or permanent based on persistence, homo- or hetero-oligomeric based on interacting surface or partners and obligate or non-obligate based on their stability (Zhang, 2009).

In this study, to understand the path of action of Ste20 and Cla4 in regulation of glucose metabolism, it was important to first identify PPI between Ste20 and Cla4 and the enzymes of glucose metabolism especially those involved in glycolysis, gluconeogenesis, pentose phosphate pathway and glycerol metabolism. The split-ubiquitin system was used to identify these PPIs. Some enzymes involved in glycolysis and gluconeogenesis (fig 1.8) such as Eno1, Tdh1, Pfk2 and Pck1, Tpi1, Glk1 showed a strong interaction with Ste20 while Fbp1 showed a very weak interaction due to the level of growth observed in the screen. For Cla4, only Tdh1 showed a strong interaction while Eno1, Tpi1, Glk1 showed a weak interaction and Fbp1, Pfk1, Pck1 showed no interaction. According to this present study, the split-

ubiquitin system used gives information about specific PPIs based on persistence with interacting surfaces due to its sensitivity in detecting weak or transient interaction and not those proteins found within a complex. The nature of such interaction could be strong/permanent or weak/transient. For those screens showing a strong/permanent interaction, they suggestively indicate they form a stable complex with Ste20 or Cla4 while those with weak or transient interaction suggestively indicate they could form a part of a signalling pathway with Ste20/Cla4 or maybe these interactions only appear weak because of the assay used. This claim of protein-protein interaction based on persistence and what their true nature of cellular engagement could be has been reported (Srinivasa Rao, et al., 2014). This implies that Ste20 and Cla4 may regulate glycolysis and gluconeogenesis by forming stable complex with some enzymes and with others, transduce pathway signals. With these results and due to the relevance of glycolysis in energy generation and generation of metabolites for synthesis of amino-acids, nucleic acids (Locasale, 2018) amongst others, it could be argued that Ste20 and Cla4 might play an important role in energy metabolism such as in regulation of several enzymes although there isn't really much evidence for a regulation.

Screens for interaction of Ste20 and Cla4 with four enzymes involved in the PPP, specifically the non-oxidative phase, Rpe1, Tal1, Sol4 and Gnd2 showed that all four enzymes interacted strongly with Ste20. For Cla4, Rpe1 and Tal1 screen showed strong interaction while Sol4 and Gnd2 due to slight growth of screened cells suggestively indicating a weak interaction. For screens showing strong interactions, it implies that Ste20 and Cla4 may form a stable complex with these enzymes while for those with weak or transient interactions they may be involved in signal transduction via Ste20 and Cla4. Based on the outcome of this screen,

it could be argued that Ste20 and Cla4 might exert an important role in the regulation of the PPP, particularly the non-oxidative phase as it provides needed and essential metabolites including nucleic acids, required by rapidly dividing cells (Cadiere, et al., 2011). Regulation of the PPP has been reported in response to oxidative stress through the generation of NADPH (Kruger, et al., 2011), a process Ste20 has also been identified to play a role in (Ahn, et al., 2005). With the outcome of this study, understanding the role Ste20 and Cla4 may play in regulation of PPP is a potentially interesting research focus.

Finally, screens for interaction with three enzymes involved in glycerol synthesis from glucose via dihydroxyacetone phosphate, Gpd1, Gpp1 and Gpp2 with Ste20 and Cla4 showed that all three tested enzymes had strong interaction with Ste20 while with Cla4, only Gpd1 and Gpp1 showed strong interaction as Gpp2 showed weak or transient interaction. This suggests that Gpd1, Gpp1 and Gpp2 may form a stable complex with Ste20 while Gpd1 and Gpp1 may form a stable complex with Cla4 and Gpp2 may be involved in a signalling cascade via Cla4. Although the role of Ste20 and Cla4 via the MAPK cascade in regulating the expression of these glycerol synthesizing enzyme under different conditions has been reported (Albertyn, et al., 1994; Mager & Siderius, 2002; Rensing & Ruoff, 2009), the direct relationship or interaction with enzymes of glycerol synthesis has not been reported. This study therefore has closed the gap in identifying the possibility of Ste20 and Cla4 in regulating these enzymes independent of the MAPK cascade.

It is important to address at this point that all the enzymes of glucose metabolism investigated in this study have been tested. Put together, this study suggests that Ste20 and Cla4 might play a broad role in glucose metabolism as they interact with several enzymes of involved in different metabolic pathways of glucose. Interestingly, a homologue of Ste20 in

human Pak1 (fig 1.1) - which is also an effector of mammalian Cdc42 - has been shown to successfully replace Ste20 function in yeast (Brown & Chant, 1996). Pak1 has been reported to inhibit phosphoglycerate mutase, PGAM, an enzyme of the glycolytic cascade hence switching from glycolysis to PPP (Shalom-Barak & Knaus, 2002). In neutrophils this leads to increased generation of NADPH resulting in an increased oxidative and phagocytic response. This further point towards growing evidence or possibilities of Ste20 and maybe Cla4 in exerting some functional roles in glucose metabolism in different capacities.

7.2 investigation of the role of Ste20 and Cla4 in Gpd1 expression

One of the focus of this study was to elucidate the biochemical role of Ste20 and Cla4 in the expression of Gpd1 under different conditions in relation to glucose metabolism. The rationale behind testing Gpd1 expression is from the established role of Ste20 and Cla4 in regulation of Gpd1 transcription via the MAPK cascade (Rensing & Ruoff, 2009) and identification of interaction with Gpd1. Gpd1 expression was tested under optimum growth condition, response to osmotic stress and response to varying glucose concentrations, all of which Ste20 and Cla4 have been reported to be involved in (Foster, et al., 2013; Chen & Thorner, 2007; Cullen & Sprague Jr., 2012; Rensing & Ruoff, 2009; Han & Ying, 2006).

Levels of Gpd1 were tested under optimal growth condition. Overexpression of *STE20* and *CLA4* had no effect on Gpd1 expression levels as they were similar to those in the wild type. Also, in single deletion strains of *STE20* and *CLA4* levels of Gpd1 were unchanged and similar to those in the wild type across all indicated time points. To this end, it was also necessary to test whether deletion of both *STE20* and *CLA4* in a single strain has any effect on Gpd1 expression level. Since deletion of both PAKs in a single strain of yeast is lethal (Cvrckova, et al., 1995), a viable strain in which *STE20* was deleted and the wild type *CLA4* is replaced by a sensitive analogue Cla4 allele, *cla4-as3,* whose kinase activity can be inhibited by the chemical inhibitor 1NM-PP1 was constructed and confirmed using sequence analysis from a previous report (Weiss, et al., 2000) and microscopic analysis of cell morphology.

Double mutation of both Ste20 and Cla4 resulted in a significant reduction of Gpd1 expression levels at all indicated time point when compared to those in the wild type. However, because in the three MAPK pathways involving Ste20 and Cla4, Ste11 is an effector MAPKKK activated by these PAKs (Rensing & Ruoff, 2009), it was important to test

levels of Gpd1 in a *Δste11* deletion strain to confirm whether the observed decrease of Gpd1 in the *Δste20 cla4-as3* double mutant strain was maybe due to a loss of induction of transcription via the Ste20/Cla4-Ste11 in the MAPK cascade or via an unidentified role of Ste20 and Cla4. Levels of Gpd1 in the *Δste11* single deletion strain were similar to those in the wild type and significantly higher than those in the *Δste20 Δcla4-cla4-as3* double mutant strain. This results clearly imply that Ste20 and Cla4 regulate the expression of Gpd1 independent of the Ste20/Cla4-Ste11 link but rather by a different mechanism, although still unclear, under optimal growth condition.

Levels of Gpd1 expression were then tested under osmotic stress condition induced with 0.8M NaCl. Gpd1 is the active form of *GPD* whose transcription is upregulated in synthesis of glycerol during response to osmostress via the Ste20 MAPK cascade (Babazadeh, et al., 2017; Rensing & Ruoff, 2009; Mager & Siderius, 2002). In single deletion strains of *STE20* and *CLA4*, levels of Gpd1 were unchanged and similar to those in the wild type. In the double mutant strain, levels of Gpd1 were similar to those of the wild type only at the point, first few minutes, of temporary repression of protein synthesis which has been reported as a mechanism that occurs due to the shock of the sudden increase in osmotic pressure (Bilsland-Marchesan, et al., 2000). After this point, levels of Gpd1 significantly reduced when compared to those in the wild type. These data imply that together, Ste20 and Cla4 regulate the expression of Gpd1 during osmoadaptation. To identify whether this observation was due to the Ste20/Cla4-Ste11 link, levels of Gpd1 were tested in *Δste11* single deletion strain and this level were similar to those in the wild type and significantly more than those in the double mutant strain. This shows that Ste20 and Cla4 regulate Gpd1 through a mechanism independent of their association with Ste11 in the MAPK cascade.

Finally, investigation of the role of Ste20 and Cla4 in response to varying glucose concentrations was performed. The rationale behind this comes from the knowledge that Ste20/Cla4 via the MAPK cascade regulate filamentous growth in response to nutrient availability such as glucose (Cullen & Sprague Jr., 2012) . In a separate study, levels of Gpd1 were unchanged under conditions that triggered filamentation, agar plate lacking glucose, when compared to agar plates with glucose. However, a remarkable difference in Gpd1 level was observed under same conditions of glucose in a liquid culture where levels of Gpd1 were significantly increased in the liquid culture with glucose. The difference in Gpd1 level between the liquid medium lacking glucose and that with glucose correlates with levels of Gpd1 under optimal growth condition where Gpd1 level was lowered in cells grown overnight (starved and glucose depleted), mimicking levels observed in liquid medium lacking glucose and an increase in Gpd1 level when these cells are transferred to a fresh glucose-rich medium, mimicking the level observed in the liquid medium with glucose.

How Ste20 and Cla4 regulate the expression of Gpd1 in response to 2% glucose was then investigated further. In the single mutant strains, expression of Gpd1 remained unchanged and similar to those of the wild type at all indicated time points. When levels of Gpd1 were tested for in the double mutant strain, they were significantly reduced compared to those in the wild type. Also, to establish whether this observed decrease was connected to the Ste20/Cla4-Ste11 link, levels of Gpd1 were tested in the *Δste11* single mutant strain and were similar to those in the wild type but significantly increased by at least a two-fold those in the double mutant strain. These data show that Ste20 and Cla4 regulate the expression of Gpd1 independent of the Ste20/Cla4-Ste11 link but rather via a different mechanism.

These data put together, clearly shows that Ste20 and Cla4 regulate Gpd1 expression via a different mechanism independent of their relationship or link with Ste11 in the MAPK cascade. Also, it shows both of them have an overlapping function which is consistent with previous reports (Cvrckova, et al., 1995).

The result of the protein-protein interaction rather suggests the formation of a stable complex between Ste20/Cla4 and Gpd1 and based on evidence, proteins involved as a complex with others tend to have a greater interaction-induced stability (Dixit & Maslov, 2013). A cycloheximide chase assay, shows Gpd1 has a half-life of about 2hrs and since in the double mutant strains, cells were exponentially grown for about 3hrs then this time is sufficient enough to allow for the destabilization and subsequent degradation of Gpd1. Also, Gpd1 level under all test conditions in the double mutant strain did not reduce as it would be expected if there was a loss of induction of transcription. Rather, Gpd1 level increased but not to a significant level as seen in the wild type. This identifies that protein synthesis is not inhibited but rather another factor accounts for the reduced levels of Gpd1 as seen in all test conditions. Protein stability remains the only factor that affects protein levels and the outcome of this study therefore strongly suggests that in addition to what is known of the role of Ste20 and Cla4 in transcriptional regulation of Gpd1 expression both PAKs seem to be directly involved in interaction-induced stabilization of Gpd1 protein and the presence of either overlaps the function of the other in this regard.

7.3 Analysis of the effect of Ste20 and Cla4 on the phosphorylation status of Gpd1

As kinases, Ste20 and Cla4 mediate their biochemical role by phosphorylating their substrates. Protein phosphorylation is the most studied form of post translational modification that plays a crucial role in the regulation of cellular metabolism, particularly glucose metabolism (Humphrey, et al., 2015). Phosphorylation affects the characteristics of proteins in a number of ways including change in enzyme activity (Portela, et al., 2006; Chen & Nielsen, 2016) , protein localisation (Jung, et al., 2010; Fernandez-Garcia, et al., 2012)and protein degradation or stability (Iwaki, et al., 2005; Swaney, et al., 2013). Gpd1 a phospho-protein, has been identified to have at least four phosphosites, S23/S24/S25/S27 (Oliveira, et al., 2012). Phospho-proteomic study of Gpd1 has shown that phosphorylation at sites S24 and S27 translocate Gpd1 from the cytosol into the peroxisome (Jung, et al., 2010; Lee, et al., 2012) while phosphorylation on any or all four phosphosites result in decrease Gpd1 activity (Oliveira, et al., 2012). The phosphorylation status of Gpd1 was tested under the same conditions for which Gpd1 expression was tested.

Under optimal conditions, levels of expressed phosphorylated and unphosphorylated proteins increased massively in the wild type while in the double mutant strain, these levels were relatively unchanged and were massively decreased when compared to those in the wild type. In response to osmostress, phosphorylated and unphosphorylated protein increased massively immediately after recovery from the brief period of osmotic shock, characterised by repression of protein synthesis, in the wild type while in the double mutant phosphorylation status was relatively unchanged after the brief period of osmotic shock and these levels were greatly reduced when compared to the wild type. Finally, in response to 2% glucose, expression level of phosphorylated and unphosphorylated proteins increased

remarkably while in the double mutant strain levels of were relatively unchanged and remarkably decreased when compared to those of the wild type. Put together, these data show that although the levels of phosphorylated proteins were remarkably reduced in the absence of Ste20 and Cla4, a closer look shows that levels of unphosphorylated proteins were also remarkably reduced. Hence, this decrease in phosphorylation status appears to be the result of the overall decrease in expression level of Gpd1

7.4 Analysis of the accumulation of glycerol

Gpd1- belongs to one of two isoforms of GPD – catalyse the rate limiting step in the synthesis of glycerol from dihydroxyacetone phosphate. Activity of Gpd1 plays a critical role in the synthesis and accumulation of glycerol under different conditions such as during osmotic stress (Lee, et al., 2012; Mager & Siderius, 2002; Nevoigt & Stahl, 1997). From results of protein expression and phosphorylation status obtained, investigating the effect of Ste20 and Cla4 in the synthesis and accumulation of glycerol was necessary. Levels of glycerol were measured intracellularly and extracellularly based on evidence that influx and efflux of glycerol freely by both simple diffusion and via dedicated Fps1 glycerol transport and Stl1 exist under different conditions and especially as a measure to control excessive accumulation of intracellular glycerol (Luyten , et al., 1995; Nevoigt & Stahl, 1997; Mager & Siderius, 2002; Ferreira, et al., 2005). Glycerol levels were measured under the same three experimental conditions of optimal growth, hyperosmotic stress and response to 2% glucose.

Under optimal growth condition, intracellular and extracellular glycerol levels, showed no marked difference in the single mutant strains of Ste20 and Cla4 in comparison to those of the wild type. It was necessary to test whether glycerol levels would reduce because of the observed significant decrease in Gpd1 levels in the double mutant strain, contrary to this, levels in the double mutant strain were found to be similar to those in the wild type and *Δste11* strain.

When levels of glycerol were checked under hyperosmotic conditions, levels of glycerol in the single mutant strains were found to be similar to those in the wild type strain. Also, from a hypothetical view point, as a result of observed significantly lesser levels of Gpd1

expression in the double mutant strain, levels of glycerol were measured to see whether this has any effect on it but this was not the outcome as levels in the double mutant strain were similar to those in the wild type and *Δste11* strains. Finally, in response to 2% glucose, levels of glycerol measured in both single mutant and double mutant strains were similar to those of the wild type.

These data put together suggests that changes in the level of Gpd1 protein does not necessarily affect glycerol levels. Possible explanations to justify these observations are 1) from the results of Gpd1 expression and phosphorylation status obtained, Gpd1 activity is not totally knocked out and reports have shown that even reduced expression and activity of Gpd1 contributes to an appreciable level of glycerol synthesis and accumulation to those in the wild type activity level (Hubmann, et al., 2011). 2) Gpd2- the second isoform of GPD – has been shown to contribute significantly to the total *GPD* activity level and glycerol level in cells with deficient or reduced Gpd1 levels (Ansell, et al., 1997).

From the data obtained in this study, it can be deduced that the presence of either PAKs overlaps with the other in regulating Gpd1 expression level while together, they exert a more definitive function by sustaining the expression levels of Gpd1. This observed effect of Ste20 and Cla4 in sustaining the expression level of Gpd1 could be explained for by the strong interaction between these proteins as obtained from the protein-protein interaction screening which suggests the possibility of the formation of a stable complex. Since stable complex proteins contribute to an interaction-induced stability, Ste20 and Cla4 may act, alone or not, as a stabilizing signalling protein on Gpd1. Hence, beyond their role in regulating transcription of Gpd1 and other genes via the MAPK cascade, they may also act in protein degradation signalling cascade. It also shows that Ste20 and Cla4 does not affect the

phosphorylation of Gpd1 which is a regulatory modification that ensures the activity level is not beyond what is required by the cell (Lee, et al., 2012). The decreased level of phosphorylation appears to be the result of reduced level of protein expression.

Chapter 8:
Conclusion and future works

8 Conclusion and Future work

8.1 Conclusion

Over the years, a reasonable number of studies have been conducted on PAKs with special interest on Ste20 and Cla4 using budding yeast as a model organism. The biology of PAKs in yeast have opened a plethora of research areas in the understanding of the role of PAKs in higher eukaryotes. A recent area in which PAKs have become interesting useful is in the study of metabolic processes such as their role has been elucidated in sterol synthesis (Tiedje, et al., 2007). In humans, PAKs have been shown to inhibit one of the enzymes of the glycolytic pathway, phosphoglycerate mutase (PGAM) (Shalom-Barak & Knaus, 2002). Findings from this study would further close the gap in the field of PAKs biology as it has been shown that the PAKs, Ste20 and Cla4, in addition to regulating gene transcription via the MAPK cascade, they also contribute to an interaction-induced stability of proteins. This can be harnessed further in tandem with previous knowledge of PAKs to improve areas such as metabolic engineering in yeast, therapeutic intervention, food and beverage production and in the understanding and tackling of fungal pathogenesis.

8.2 Future works

It will be interesting to further close the gap in knowledge in the biology of PAKs, but time is limited, and I could only get this far. To further consolidate this study here are some work that would be of interest to carry out;

1. For glucose metabolism it could be tested whether PAKs; a) regulate flux rates, b) phosphorylate key enzymes, c) affect enzyme activity or stability, d) and under which conditions this usually happens.
2. To investigate whether Ste20 and Cla4 regulate the expression of Gpd2 since it contributes to the overall GPD activity in yeast cell and overall activity level of *GPD*. To also measure Gpd enzyme activity.
3. Does the deletion of Gpd2 under the present study affect the level of glycerol accumulation since Ste20 and Cla4 have been shown to regulate the expression of Gpd1 through protein stabilization? This study could include the conditions where only one of each *GPD* isoforms are expressed. It might be worth testing how Ste20 and Cla4 activity for these processes is regulated.
4. To better understand the role of Ste20 and Cla4 in MAPK pathway, this study has only shown that Ste20 and Cla4 are independent of Ste11 in protein stability, it might be important to investigate further whether this role is independent of the MAPKK and MAPK proteins, Pbs2 and Hog1 respectively.
5. The role of Ste20 and Cla4 can be investigated in the regulation of the pentose phosphate pathway- it serves as an important supply of metabolites for cancer development and progression in human. B) it can also be tested whether the role of PAKs in this process is conserved.

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