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Author(s)	Laviña, Walter Alvarez
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Doctoral Dissertation

Multi-level functional redundancy mechanisms of *Saccharomyces cerevisiae* in response to high extracellular calcium stress

Walter Alvarez Laviña

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Graduate School of Engineering,
Osaka University

Abstract

Upon the completion of the Yeast Genome Project in 1996, *Saccharomyces cerevisiae* became the first eukaryotic genome to be sequenced fully; defining 5,885 potential protein-encoding genes. More importantly, the complete sequence revealed the magnitude of research that was needed to be done as more than 50% of the sequenced genes were unknown and uncharacterized by previously established methods such as mutant hunting. Furthermore, the yeast genome showed a large amount of genes with apparent genetic redundancy. This particular problem necessitated the need to systematically characterize the biological function of the all genes in the yeast genome and to elucidate functional redundancy in more detail.

Sakumoto et al. (2002) have previously reported that the disruption of two protein phosphatase genes, *PTP2* and *MSG5*, caused calcium sensitivity indicating that functional redundancy exists between the two protein phosphatases in response to high extracellular calcium. Furthermore, Hermansyah et al. (2010) found that additional disruption of protein kinases *BCK1, MKK1, SLT2, MCK1, YAK*1 and *SSK2* in the *ptp2*Δ*msg5*Δ background conferred calcium tolerance. In this work, the suppression mechanisms conferred by the different protein kinase disruptions are described. First, it was found that the inactivation of calcineurin by the disruption of *CNB1* or treatment with FK506 can suppress the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant. In the wake of a calcium-induced, calcineurin-driven signaling pathway activation, the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant can be suppressed by regulating the SLT2 pathway through the disruption of the major kinases in the SLT2 signal cascade that include *BCK1, MKK1* and *SLT2.* Also, this study showed that *PTP2* and *MSG5* are key regulatory phosphatases that prevent over-activation of the calcium-induced signaling cascade under the parallel control of the SLT2 and calcineurin pathways. Next, genetic analysis showed a HOG-pathway

independent function for Ssk2 in response to high extracellular calcium. Genome-wide transcriptional analysis found 19 induced genes in the calcium-sensitive *ptp2*Δ*msg5*Δ double disruptant that were subsequently repressed in the calcium-tolerant *ptp2*Δ*msg5*Δ*ssk2*Δ triple disruptant ("rise and fall" patterned expression). Also, this study reports new suppressors, *bcy1*Δ and *msn2*Δ, which are related to the previously determined suppressors *ssk2*Δ and *yak1*Δ via the regulation of the "rise and fall" genes. This study provides evidence to the existence of several alternative pathways that mediate calcium signaling in the *ptp2*Δ*msg5*Δ double disruptant.

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Chapter 1

General Introduction

1.1 The Yeast Genome Project and functional redundancy in yeasts

In 1990, the Human Genome Project (HGP) was initiated by the US Department of Energy (DOE) and National Institutes of Health (NIH) with the primary goal of sequencing, identifying and mapping the entire 20,000 to 25,000 genes of the human genome (www.genome.gov). Several genome projects paralled from this international and collaborative research initiative using model organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila* sp. and *Arabidopsis thaliana* (www.nigms.nih.gov). In 1996, the genome of the yeast *S. cerevisiae* was completely sequenced with 12,068 kilobases defining 5885 potential protein-encoding genes, (including 140 ribosomal RNA genes, 40 genes specifying for small nuclear RNA molecules and 275 transfer RNA genes). (Goffeau et al., 1996). Aside from the fact that the *S. cerevisiae* was the first eukaryotic genome to be fully sequenced, yeast has been considered an ideal model organism to study biological phenomena due to its intrinsic advantages as an experimental system such as an ideal life cycle perfectly suited for genetic analysis and ease of genetic manipulation.

Although *S. cerevisiae* has been one of the best characterized organisms from the genetics and physiological point of view, results of the Yeast Genome Project revealed the magnitude of research that was still needed to be done as more than 50% of the sequenced yeast genes were unknown and uncharacterized by previously established methods such as mutant hunting (Piskur and Langkjaer, 2004). Such considerable amount of uncharacterized genes was accounted to genetic redundancy, which was believed to be responsible for cellular robustness (Goffeau et al., 1996; Li et al., 2010). This unexpected discovery necessitated the

need to systematically characterize the biological function of the all genes in the yeast genome and to elucidate functional redundancy in more detail.

1.2 Cellular response to environmental perturbation and stress

The ability to respond to changes in environmental conditions for the purpose of cell protection or damage repair is an intrinsic property of the cell, regardless whether it is prokaryotic or eukaryotic. To achieve this, cells require a complex network of sensory and signal transduction mechanisms that lead to adaptations in cell growth and proliferation, usually by modification in gene expression and metabolic activities (Hohmann and Mager, 2002). Specifically, stress refers to the state of environment that is not optimal and often detrimental to the survival of cells. When cells encounter drastic changes in the environments, survival largely depends on maintaining cellular homeostasis thus, cells developed various ways of keeping the cellular state in equilibrium. For example, in the advent of stress exposure that affect the cell wall, a transcriptional response consisting of the general stress response, cell wall integrity and the calcineurin pathway is observed (Klis et al., 2006). Conversely, it is also essential for the cells to possess alternative “fail-safe” mechanisms in case of improper activation of these pathways that can be detrimental to the organism.

1.3 Reversible phosphorylation dynamics (Protein kinase and phosphatases)

Cell signaling plays a very crucial role in the survival of organisms where a slight change in the cell dynamics can be detrimental. Hence, organisms evolved to have several parallel pathways to prevent unwanted activation or deactivation of signaling cascades. Furthermore, each signaling cascade possesses several mechanisms of regulation, ranging from transcriptional to post-translational. Reversible phosphorylation is one among the many regulatory mechanisms that cells employ to mediate cell signaling. Under this regulation,

proteins are phosphorylated with protein kinases (PKases) while protein phosphatases (PPases) are responsible for dephosphorylation. This process plays a key role in adaptation to environmental stress and cell proliferation (Martin et al., 2005).

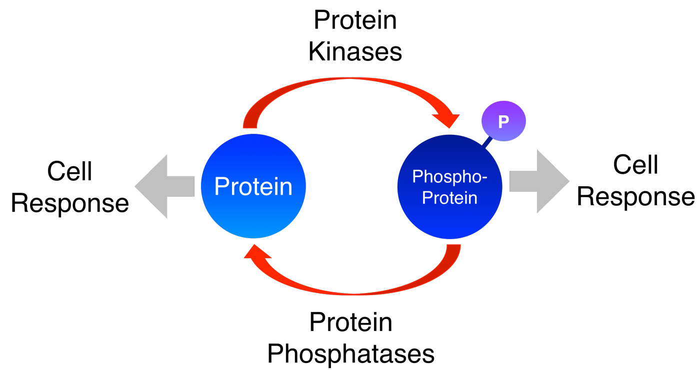

Figure 1. Reversible phosphorylation dynamics in cells

In *S. cerevisiae*, there are 129 PKases and 40 PPases among approximately 6,600 genes that are involved in various aspects of signaling and other cellular aspects (www.yeastkinome.org; Sakumoto et al., 2002). Yeast protein phosphatases are divided into four groups namely: 1) serine threonine PPases, 2) protein tyrosine PPases, 3) dual specificity PPases and PTEN and myotubularin PPases. These groups of proteins have their corresponding homologs in humans and other higher eukaryotes although the number in each group is much larger.

Table 1. Protein phosphatase (PPase) genes present in *S. cerevisiae*

Family/ Subfamily	ORF/ Gene	Function
PPP family		
PP1 subfamily	*YER133w/GLC7*	Involved in glycogen metabolism, sporulation and mitosis
	YML016c/PPZ1	Involved in salt homeostasis
	YDR436w/PPZ2	Involved in salt homeostasis
	YPL179w/SAL6	Regulation of protein synthesis
PP2A subfamily	*YDL047w/SIT4*	Cell cycle
	YDL134c/PPH21	Cell cycle, cell wall maintenance
	YDL188c/PPH22	Highly homologous to Pph21p
	YDR075w/PPH3	Cell structure
	YNR032w/PPG1	Involved in glycogen accumulation
PP2B subfamily	*YLR433c/CNA1*	Some ion transport, cell polarity, mating response
	YML057w/CNA2	Some ion transport, mating response
Other	*YGR123c/PPT1*	Human PP5-related PPase
	YLL010c/PSR1	Response to stress
	YLR019w/PSR2	Functionally redundant Psr1p homolog
	YMR277w/FCP1	Carboxy-terminal domain (CTD) phosphatase
	YLR361c/DCR2	Interacts with Sic1p, an inhibitor of mitotic cyclin/cyclin-dependent kinase complexes.
	YHR004c/NEM1	Regulates nuclear growth
	YNL222w/SSU72	Phosphatase and transcription/RNA processing factor
PPM family		
PP2C subfamily	*YDL006w/PTC1*	Down-regulate the HOG pathway
	YER089c/PTC2	Cdc28p dephosphorylation, involved in HOG pathway
	YBL056w/PTC3	Cdc28p dephosphorylation, Involved in HOG pathway
	YBR125c/PTC4	Cytoplasmic type 2C PPase
	YOR090c/PTC5	Involved in regulation of pyruvate dehydrogenase activity
	YCR079w/PTC6	Mitochondria type 2C PPase with similarity to mammalian PPk1s
	YHR076w/PTC7	Mitochondrially localized type 2C PPase

PTP family		
PTP subfamily	*YDL230w/PTP1*	Protein tyrosine phosphatase
	YDR208w/PTP2	Down-regulate Hog1p and Fus3p MAPK, sporulation
	YER075c/PTP3	Down-regulate Hog1p and Fus3p MAPK, sporulation
	YPR073c/LTP1	Similar to PPase from human placenta
	YNL032w/SIW14	Involved in nutritional control of the cell cycle
	YMR036c/MIH1	*S.pombe* cdc25p homologue
	YJR110w/YMR1	Phosphatidylinositol 3-phosphate phosphatase
	YNL128w/TEP1	Similar to human tumor suppressor gene
DSP subfamily	*YFR028c/CDC14*	Function at the late stage of the cell cycle
	YBR276c/PPS1	A role in the DNA synthesis phase of the cell cycle
	YIR026c/YVH1	Yeast homologue of Vaccinia virus PTP, VH1
	YNL053w/MSG5	Dephosphorylate Fus3p
	YIL113w/SDP1	Negatively regulates Slt2p MAPK
	YNL099c/OCA1	Protein tyrosine/serine/threonine phosphatase activity
Other	*YNR002c/ATO2*	Weak similarity to PPase

Elucidation of the protein functions of PPases and PKases contribute greatly to the understanding of various signaling pathways in the cell, paving the way to the clarification of the nature of diverse unknown cellular phenomena. Although knowledge about PKases and PPases has steadily grown over the past years, there are still a fair number of PPases that needs to be addressed. Between the two proteins, analysis of the functional redundancy of PPases seemed more practical thus it was undertaken. In our laboratory, a library of 30 PPase single disruptants and double disruptants in all 435 possible combinations was created to identify the role of every phosphatase gene in various cellular functions of *S. cerevisiae.* Systematic genetic screening led to the discovery of several interesting phenotypes such as temperature sensitivity, caffeine sensitivity and cation sensitivity. Through this work, we found that disruption of both *PTP2* and *MSG5* in *S. cerevisiae* causes calcium sensitivity, while single disruption of *PTP2* or *MSG5* does not (Sakumoto *et al*., 1999 & 2002). The synthetic phenotype displayed by the *ptp2*Δ*msg5*Δ double disruptant suggests a functional redundancy existing between *PTP2* and *MSG5* in response to high extracellular calcium stress. We were intrigued with the first report of the involvement of redundant function of established MAPK regulators, Ptp2 and Msg5, in the calcium-induced signaling pathway

thus, the calcium sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant was further studied.

1.4 Ptp2 and Msg5 protein phosphatases

PTP2 (*YOR208W*) encodes a 750-amino acid residue, 86 KDa protein tyrosine phosphatase that has 36-46% amino acid sequence similarity with at least 35 mammalian PPases that includes *PTPRC* (PTP receptor type T) and *PTPRT* (PTP receptor type T) (Guan et al., 1992; Wang et al., 2004). On the other hand, *MSG5* (*YNL053W*) codes for a 489-amino acid residue, 54 KDa dual-specificity protein phosphatase that has 42-72% amino acid sequence similarity with at least 27 mammalian PPases that includes *MKP5*, *DUSP26* and *DUSP3* (Guan et al., 1992; Rahmouni et al., 2006). Although Ptp2p and Msg5p are both included in the PTP family of PPases and contain an active-site signature motif HCX5R, Ptp2p dephosphorylates phosphotyrosine residues while Msg5 acts on both phosphoserine/phosphothreonine and phosphotyrosine residues (Guan et al., 1992; Doi et al., 1994; Sakumoto et al., 1999; 2002). Previous genetic and biochemical studies implicated protein tyrosine phosphatases (PTPases) with the negative regulation of MAP kinases (Zhan et al., 2000). Conventionally, Ptp2*,* a phosphotyrosine-specific PPase, is important in the inactivation of Hog1 MAPK during high osmolarity (Wurgler-Murphy *et al*., 1997) and heat stress (Winkler *et al*., 2002) while Msg5*,* a dual-specificity PPase, is involved in the regulation of the mating response pathway (Doi *et al*., 1994; Blackwell *et al*., 2007) and cell integrity pathway (Flandez *et al*., 2004). However, there has been evidence that *PTP2* and *MSG5* also play important roles in calcium signaling in yeasts (Sakumoto et al., 1999).

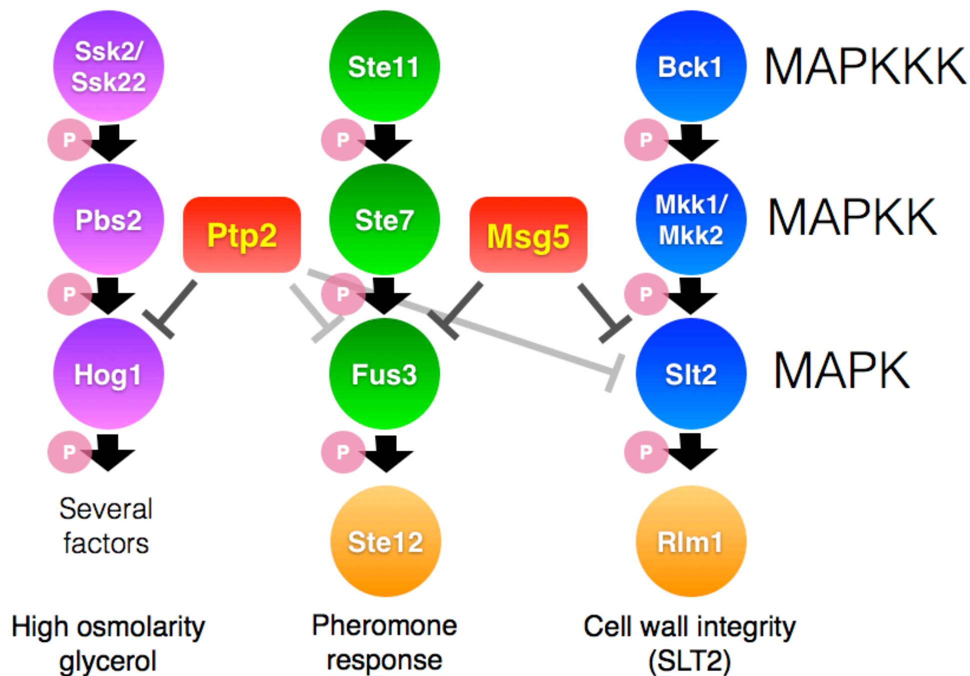

Figure 2. MAPK cascades in *S. cerevisiae* (Adapted from Martin et al., 2005)

1.5 Calcineurin and calcium homeostasis

Adaptation to changing environments require cells to activate signaling pathways that require messengers whose concentration varies with time (Clapham, 2007). Calcium ion, which is the most ubiquitous and versatile signaling molecule in the eukaryotic cell, fit this specific role perfectly thus making it arguably the most important signaling molecule in the cell. In *S. cerevisiae*, exposure to calcium activates the Ca^{2+}/ calmodulin-dependent protein phosphatase calcineurin that regulates the Crz1p transcription factor (Yoshimoto et al., 2002). Calcineurin is a heterodimeric protein comprised of a catalytic (A) and regulatory (B) sub-units that mediates the nuclear localization of the transcription factor Crz1. Furthermore, calcineurin is specifically inhibited by immunosuppressant drugs cyclosporin A and tacrolimus (FK506) thus making it a very valuable protein for clinical studies (Sugiura et al., 2002; Cyert 2003). Under normal conditions when cytosolic Ca^{2+} levels are low, calcineurin is inactive but in response to stress, there is a rise in the cytosolic Ca^{2+} levels that activates calcineurin, subsequently dephosphorylating several key proteins to promote survival (Cyert, 2003). One important function of calcineurin-dependent signaling is the

regulation of the transcription factor Crz1. Crz1p directs a calcium-induced, calcineurin dependent gene expression by binding to calcineurin-dependent response element (CDRE) (Stathopoulos et al., 1997). Calcineurin-induced activation leads to up-regulation of 163 genes including *GSC2*, *PMC1*, *PMR1* and *ENA1* (Yoshimoto et al., 2002). Calcineurin and Crz1-regulated genes belong to various functional classes such as integral membrane proteins, plasma membrane and cell wall components, lipid synthesis, protein degradation and vesicle trafficking (Cyert, 2003).

1.6 SLT2 pathway

The Slt2/Mpk1 mitogen-activated protein kinase (MAPK) cell integrity pathway is responsible for the monitoring and proper response to stresses that challenge the integrity of the cell wall as well as the maintenance of cell shape and integrity during vegetative growth and mating in *S. cerevisiae* (Jung and Levin, 1999; Hahn and Thiele, 2002). Slt2 pathway sensors Wsc1 and Mid2 interact with Rom2 to trigger Rho1 GTP loading which then activates Pkc1. Afterwards, there is a sequential activation of the MAPK cascade consisting of MAPK kinase kinase Bck1, MAPK kinase Mkk1/Mkk2 and MAP kinase Skt2 (Cid et al., 1995; Levin , 2005).

1.7 HOG pathway

Upon exposure to increased osmolarity, S. cerevisiae responds by activating the High Osmolarity Glycerol (HOG) MAPK pathway. The cascade is made up of three sequenitially activating kinases; the redundant MAPK kinase kinases, Ssk2 and Ssk22, MAPK kinase Pbs2 and the MAP kinase Hog1 (Saito and Tatebayashi, 2004). Three upstream, independent osmosensors regulate the HOG pathway namely the membrane-spanning proteins Sln1p and Sho1p and a third Msb2 branch (O'Rourke and Herskowitz, 2002). The Sln1 transmembrane

protein contains a cytoplasmic histidine kinase (HK) and receiver (Rec) domains and has strong similarities with the bacterial two-component signal transducers (Saito and Tatebayashi, 2004). The phosphate is transferred from Sln1 to Ypd1 via a phospho-relay mechanism until it reaches another Rec domain protein, Ssk1 (Posas et al., 1996). Upon exposure to hyper-osmotic conditions, Ssk1 is rapidly dephosphorylated by Ypd1 and binds the functionally redundant Ssk2/Ssk22 MAPKK kinases. Ssk2/Ssk22 in turn activates Pbs2 MAPKK. On the other hand, the SHO1 branch involves a transient formation of cell surface protein complexes consisting of at least Sho1p and Pbs2p (Reiser et al., 2000). Cdc24, Cdc42, Ste20, Ste50 and Ste11 are also reported to have involvement in this signaling cascade (Hohmann, 2002). The signals from each branch converge to Pbs2 which in turn activates Hog1. Activation of the key element Hog1 through the phosphorylation of the conserved Thr174 and Tyr176 residues results to a change in the transcriptional pattern in response to osmotic stress; which includes the expression of several genes such as *GPD1*, *CTT1*, and *HSP12* (Wurgler-Murphy et al., 1997).

1.8 cAMP-protein kinase A Pathway and Yak1

In *S. cerevisiae*, cAMP-Protein Kinase A (PKA) pathway plays a major role in the regulating cell growth, metabolism and stress resistance (Tamaki, 2007). PKA is essential for cell cycle progression since the absence of PKA activity results in cell arrest at G1 and growth inhibition (Garrett et al., 1991). PKA is composed of a catalytic sub-unit encoded by three genes (*TPK1, TPK2* and *TPK3*) and a regulatory sub-unit encoded by *BCY1* (Thevelein and de Winde, 1999). Although the main function of the cAMP-PKA pathway is for nutrient sensing, it was also reported to control the expression of around 30 STRE (stress-responsive element) genes via the repression of the transcription factors Msn2 and Msn4 (Wilson and Roach, 2002).

YAK1 encodes for a serine-threonine protein kinase and a known component of the glucose-sensing system initially identified as a suppressor of the lethal phenotype associated with loss of RAS function or loss of the catalytic unit of PKA (Pratt et al., 2007). Yak1 functions downstream of PKA and is a negative regulator of growth.

1.9 Environmental Stress Response (ESR) and transcription factors Msn2 and Msn4

Due to the constant fluctuations in environmental conditions, cells are prone to changes in their internal milieu that can disrupt normal cellular processes. Therefore, cells must maintain their internal system at a stable state to maintain viability. A mechanism employed by yeast to protect their internal system from drastic changes is the environmental stress response (ESR). ESR includes approximately 900 genes that are normally altered transcriptionally when exposed to a stressful environment (Martinez-Pastor et al., 1996; Gasch, 2002). Specifically, ESR response consists of around 300 induced genes related to stress response and 600 repressed genes related to protein synthesis (Berry and Gasch, 2008). Although the simultaneous expression of this group of genes is the main characteristic of ESR, regulation of the gene expression is gene-specific and condition-specific (Gasch, 2002).

The best characterized regulators of ESR gene expression are the transcription factors Msn2p and Msn4p. These transcription factors specifically bind to the STRE promoter sequence and induce the transcription of ESR genes in response to stress. Even though both factors were initially thought to be redundant, Msn2 and Msn4 appear to respond differently based on the genotype and condition with Msn2 being regarded as the stronger transcription factor (Hohmann, 2002). Furthermore, results by Berry and Gasch (2008) indicated that Msn2 and Msn4 play distinct roles in response to various types of stresses. However, both Msn2 and Msn4 are required by many genes for full induction (Berry and Gasch, 2008).

1.10 Objective of the study

In an effort to study the functional redundancy of protein phosphatases in yeast, Sakumoto and colleagues (2002) constructed a PPase library of single and double disruptants and systematically screened for interesting phenotypes. One such phenotype was the calcium sensitivity exhibited by the strain with the disruption of both *PTP2* and *MSG5* but not the single disruptants *ptp2*Δ or *msg5*Δ (Sakumoto et al., 1999; 2002). Furthermore, disruption of an additional protein kinase in the *ptp2*Δ*msg5*Δ double disruptant revealed *bck1*Δ*, mkk1*Δ and *slt2*Δ of the Cell Wall Integrity pathway*, mck1*Δ which is involved in chromosome segregation and meiosis, *yak1*Δ of the glucose sensing pathway and *ssk2*Δ of the HOG pathway as suppressors of the calcium sensitive phenotype (Hermansyah et al., 2010). Interestingly, since the PKase suppressors belong to various signaling pathways, this indicates that the Ptp2 and Msg5-mediated calcium signaling pathway involves a complex interaction between various signal cascades. Thus, the purpose of this study is to elucidate the suppression mechanism/s of the calcium-sensitivity of the *ptp2*Δ*msg5*Δ double disruptant conferred by the different PKase suppressors and discover their interrelationships. In addition, we searched for other genes that may be related to the calcium-induced signaling mediated by PPases Ptp2 and Msg5.

In Chapter 1 of this dissertation, the importance of cell signaling and reversible phosphorylation on the cell's response to the environment was described. In addition, several important signaling cascades are outlined namely the calcineurin, SLT2, HOG and PKA pathways. In Chapter 2, the suppression of the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant via the inactivation of calcineurin either by the disruption of the calcineurin regulatory subunit, *CNB1* or treatment with a calcineurin inhibitor, FK506 was described. Also, evidence of a functional redundancy at the cascade level between SLT2 and calcineurin pathways in response to high extracellular calcium was shown. In this model,

the roles of Bck1, Mkk1 and Slt2 in the calcium-induced, Ptp2 and Msg5-mediated signaling was clarified. Chapter 3 described two new suppressors, *msn2*Δ and *bcy1*Δ that are genetically related to the previously identified suppressor *ssk2*Δ via the regulation of a specific gene cluster. Moreover, a novel, HOG-independent suppressor function of Ssk2 and 19 genes with "rise and fall" pattern of expression that is likely involved in the calcium phenotype of the *ptp2*Δ*msg5*Δ double disruptant were identified. Results suggest the interrelationship of a HOG-independent function of Ssk2, transcription factor Msn2, PKA-related protein Bcy1 and 19 "rise and fall" genes as responsible for the suppression mechanism of the *ptp2*Δ*msg5*Δ double disruptant by *ssk2*Δ disruption. The conclusion and general discussion was described in Chapter 4.

Chapter 2

Functionally redundant protein phosphatase genes *PTP2* and *MSG5* co-regulate the calcium signaling pathway in *Saccharomyces cerevisiae* upon exposure to high extracellular calcium concentration

2.1 Introduction

Upon exposure to stress, *S. cerevisiae* employs a variety of signaling cascades such as the mitogen-activated protein kinase (MAPK) pathways that control patterns of gene expression and protein activity to cope with the deleterious changes in the environment (Gustin et al., 1998). The ability of the cells to correctly perceive and respond to the environment is critical to the organism's survival thus; inappropriate activation of cell signaling pathways often has dire consequences on the viability of the cell. Accordingly, regulation is an integral part of the signaling cascade architecture that ensures the maintenance of cellular homeostasis upon fluctuations in environmental conditions. Since over-activation of a particular signal can have damaging effects, cells possess a myriad of ways to combat improper activation of signaling pathways, one of which is regulation via protein phosphorylation and dephosphorylation catalyzed by protein kinases (PKases) and protein phosphatases (PPases) respectively.

In *S. cerevisiae*, high extracellular levels of calcium usually elicit the activation of calcineurin, a calcium/calmodulin-dependent serine/threonine PPase (Cyert, 2003). Calcineurin is known to regulate Ca^{2+} pumps and exchangers responsible for calcium homeostasis in yeast to maintain the cytoplasmic concentration in the range of 100-300 nM (Cunningham and Fink, 1994; Davis, 1995). These include the vacuolar H^{+} ATPases encoded by *VCX1* (5), vacuolar Ca^{2+} ATPase Pmc1p (Cunningham and Fink, 1994) and high affinity Ca^{2+}/Mn^{2+} P-type ATPase Pmr1p (Rudolph et al., 1989). A rigid control of the

intracellular Ca^{2+} concentration is an important feature of calcium signaling in which transient alteration of the cytosolic calcium concentration leads to the activation of several signal transduction pathways (Muller et al., 2001). In the regulation of these signaling pathways, calcineurin plays various physiological roles including recovery from α-factor-induced growth arrest, salt and temperature tolerance, calcium and ion homeostasis, regulation of cell wall biogenesis and Mn^{2+} tolerance (Stark, 1996; Miyakawa and Mizunuma, 2007).

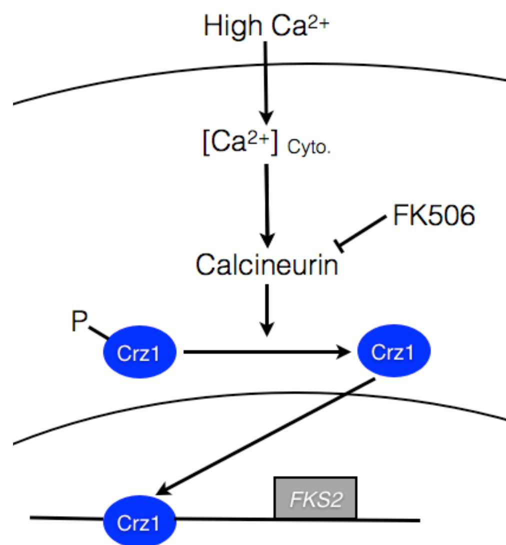

Fig. 3. Calcineurin/Crz1 signaling pathway in *S. cerevisiae* (Adapted from Yoshimoto et al., 2002)

We have previously reported that the disruption of two PPases namely *PTP2* and *MSG5* has a detrimental effect on the growth of yeast in a calcium-rich medium. Furthermore, the fact that *ptp2*Δ and *msg5*Δ single disruptants were calcium tolerant implies that there is functional redundancy existing between the two PPases in response to high extracellular calcium (Sakumoto et al., 2002). The calcium-sensitive *ptp2*Δ*msg5*Δ double disruptant was further described to exhibit G1 delay, reduced *CLN2* expression and vacuole fragmentation (Hermansyah et al., 2009). In addition, we identified six PKases namely *BCK1, MKK1, SLT2, MCK1, YAK1* and *SSK2* whose additional disruption in the *ptp2*Δ*msg5*Δ double

disruptant background confers calcium tolerance (Hermansyah et al., 2010). Interestingly, these suppressors fall into two groups based on their capacity to restore cell cycle progression as shown by FACS analysis. Disruption of *BCK1, MKK1, SLT2* or *MCK1* in the *ptp2*Δ*msg5*Δ double disruptant was unable to alleviate the G1 transition defect while *SKK2* or *YAK1* disruption were able to initiate G1 to S transition, implying that there are at least two mechanisms governing the suppression of the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant. Although Msg5 and Ptp2 are known to be regulators of the *SLT2* pathway (Martin et al., 2005), their specific involvement in the calcium-induced signaling related to growth is not yet fully understood.

In this chapter, we show the importance of the functionally redundant *PTP2* and *MSG5* in the growth of *S. cerevisiae* in high extracellular calcium conditions. Furthermore, we found another suppressor of the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant, *cnb1*Δ, which for the first time implicates the calcineurin pathway in the calcium-induced signaling pathway regulated by the functionally redundant *PTP2* and *MSG5*. We also describe how growth is inhibited in the calcium-exposed *ptp2*Δ*msg5*Δ double disruptant in relation to calcineurin and SLT2 pathways. Lastly, we explain the mechanism of suppression conferred by the previously discovered suppressors (*bck1*Δ*, mkk1*Δ and *slt2*Δ) of the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant in relation to the redundant regulatory function exhibited by SLT2 and calcineurin pathways in cell processes related to growth.

2.2 Materials and methods

2.2.1 Strains and media

Yeast strains used in this study are listed in Table 2. FY833 or SH5209 (*MAT*a *ura3-52 his3-Δ200 leu2Δ1 lys2Δ202 trp1Δ63*) was used as the wild type strain. All experiments using yeast strains were conducted at 30°C using standard recipes for YPDA (Sigma-Aldrich Co., St. Louis, MO, USA), SC dropout and Sporulation medium (SPM). YPDA was supplemented with 0.6 M $CaCl_2$ for the calcium phenotype test while 0.3M $CaCl_2$ was used for total RNA isolation. For the calcineurin phenotype test, YPDA was added with 1 μg/ml FK506 (a calcineurin inhibitor). *Escherichia coli* strains used as sources of plasmids namely BYP1804 for p1804 (*CgHIS3*), BYP1805 for p1805 (*CgTRP1*), BYP1419 for p1807 (*CgLEU2*), SHB3276 for p3276 (*ScURA3*), BYP2446 for p2446 (*GFP-CgTPRP1*) were acquired from NBRP, YGRC, Japan and cultivated in LB medium containing 100 ⌈g/ml ampicillin at 37°C as previously described (Sambrook et al., 1989). *CRZ1* gene was tagged with GFP using the PCR-mediated gene tagging method described by Gerami-Nejad et al. (2001) with some modifications. Briefly, the *CRZ1-GFP-CgTRP1* tagging cassette for transformation of the wild type and *ptp2*Δ*msg5*Δ double disruptant strains was constructed using primers CRZ1-GFP F: 5' -GTACGAA GAAGCCAGACAGGAGAAATCGGGAC AAGAGAGTTAA-3' and CRZ1-GFP R: 5'-AAAAAAAATTCCTATTCAAAGCTTAA AAAAACAAAAATAAAAAACGACGGCCA GT-3', corresponding to 40 base pairs upstream and downstream of the *CRZ1* gene stop codon. The plasmid p2446 (containing the GFP-CgTRP1 fragment) from BYP2446 was used as template for PCR.

2.2.2 Genetic Manipulations

Construction of single, double and triple disruptants was carried out using the PCR-mediated gene disruption method (Gietz and Schiestl, 2007) using *Candida glabrata HIS3*, *C. glabrata LEU2* or *S. cerevisiae* genes (*CgHIS3*, *CgLEU2* or *ScURA3*, respectively) or mating as previously described by Hermansyah. (2009; 2010). Confirmation PCR using specific primers was used to verify the correct disruption of genes. The primers for used in this study are listed in the Table 3.

2.2.3 RNA isolation

RNA was isolated using the hot phenol method (Spellman et al., 1998) with some modifications. Briefly, cells grown to mid-log phase at 30°C in YPD with or without 0.3M $CaCl_2$ were harvested by centrifugation. Total RNA was extracted by sequential treatment of TES/distilled water (DW)-saturated phenol, Trizol-LS (BRL), chloroform, and isopropanol before precipitation with Sodium Acetate (pH 5.2) – 99.9% EtOH (DEPC) mixture. The resulting RNA was dissolved in pre-chilled distilled water.

2.2.4 Microarray analysis

Total RNA was prepared using the hot phenol method (Spellman et al., 1998) with some modifications. First-strand cDNA targets were synthesized using Amino Allyl MessageAmp™ II aRNA amplification kit (Ambion, Applied Biosystems) after which, they were labeled with Cy3-dUTP or Cy5-dUTP (Amersham Biosciences AB, Uppsala, Sweden). For DNA microarray, 3D-Gene™ Yeast Oligo chip *S. cerevisiae* 6k containing 5,888 spotted genes was used (DNA Chip Research, Inc., Yokohama, Japan) and hybridization was done using the dual color method. Microarrays were scanned with ScanArray Lite (PerkinElmer Inc., Waltham, Massachusetts, USA). Differential expression analysis of the microarray

data was carried out using Genowiz^{TM} 4.0 microarray data analysis software (Ocimum Biosolutions,Hyderabad, India). Replicated values for genes were merged and the median values of the expression ratios were considered for the dataset while empty spots were removed by filtering. Fold change analysis was done to detect highly expressed genes. Genes with 2 folds up/down-regulation were considered as differentially expressed at a p-value < 0.05, Student's t-test. Functional classification of the genes was performed using gene ontology and pathway analysis (GOstat). The list of calcium-regulated calcineurin-dependent genes considered was taken from the genome-wide gene expression studies conducted by Yoshimoto and colleagues (Yoshimoto et al., 2002).

2.2.5 mRNA level determination using quantitative PCR

The differential expression data of selected genes were validated by quantitative RT-PCR. Using the total RNA isolated by the hot phenol extraction, first strand cDNA synthesis was carried out using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems AB, Uppsala, Sweden). The synthesized cDNA was used as template for real-time PCR. Quantitative PCR was performed using 20 µl reaction mixtures on an Applied Biosystems 7300 Real Time PCR System with SYBR® Green PCR Master Mix (Applied Biosystems AB, Uppsala, Sweden). PCR was performed using the following amplification program: initial denaturation for 10 minutes at 95ºC, followed by 40 cycles of denaturation for 15 seconds at 95ºC and annealing for 1 minute at 60ºC. *ACT1* was used as the reference gene for normalization of mRNA levels. The RT-PCR primers used in this study were designed using Primer Express version 2.0 (Applied Biosystems AB, Uppsala, Sweden) and are listed in Table 4.

2.2.6 Phenotypic analysis

Sensitivity of the strains to calcium and FK506 was assessed by spot plating on solid media. Cells suspended in water at 1 X 10^6 cells/ml were spotted onto YPDA and YPDA with 0.6 M CaCl2 and 1 µg/ml FK506. The plates were incubated at 30ºC for 2 – 4 days.

2.2.7 Fluorescence microscopy

Cells expressing GFP-tagged Crz1 proteins were cultured in YPD medium with or without 0.3 M $CaCl_2$ at 30ºC until the mid-log phase after which the intracellular localization of the GFP-tagged protein was observed using a fluorescence microscope (BX61, Olympus) and photographed using a CCD camera (CCD-EX1, Universal Imaging Co., USA). DNA was stained for fluorescence using 4',6-diamidino-2-phenylindole (DAPI).

Table 2. *S. cerevisiae* strains used in the study

Strains	Alias	Genotype	Source/Description
SH6314	BY4742	*MAT* α *his3 Δ1 leu2 Δ0 lys2 Δ0 ura3Δ0*	Invitrogen
SH5209	BY5209	*MAT* a *ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	NBRP, YGRC*
SH5210	BY5210	*MAT* α *ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	NBRP, YGRC*
SH5406	BY5210 *CRZ1-GFP*	*MAT* α *ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63 CRZ1-GFP::CgTRP1*	Trp^+ transformant of SH5210 with *CRZ1-GFP* cassette
SH6790	*ptp2Δ*	*MAT* a *Δptp2::CgHIS3 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	SH5209 disruptant
SH6791	*msg5Δ*	*MAT* α *Δmsg5::CgLEU2 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	SH5210 disruptant
SH6792	*ptp2Δ msg5Δ*	*MAT* α *Δptp2::CgHIS3 Δmsg5::CgLEU2 ura3-52 his3- Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	(SH6790 x SH6791)-1B
SH6793	*ptp2Δ msg5Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	(SH6790 x SH6791)-2B
SH8966	*ptp2Δ msg5Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	Leu^+ disruptant of SH6790 single disruptant using p1807 (*CgLEU2*)
SH8971	*ptp2Δ msg5Δ CRZ1-GFP*	*MAT* α *Δptp2::CgHIS3 Δmsg5::CgLEU2 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63 CRZ1-GFP::CgTRP1*	Trp^+ transformant of SH6792 with *CRZ1-GFP* cassette
SH8928	*cna1Δ*	*MAT* α *Δcna1::KanMX4 his3 Δ1 leu2 Δ0 lys2 Δ0 ura3Δ0*	Invitrogen
SH8933	*ptp2Δ msg5Δ cna1Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 Δcna1::KanMX4 ura3-52* (or *ura3 Δ0*) *his3-Δ200* (or *his3 Δ1*) *leu2Δ1* (*leu2 Δ0*) *lys2Δ202 (lys2 Δ0) trp1Δ63*	(SH6793 x SH8928)-4C
SH8929	*cna2Δ*	*MAT* α *Δcna2::KanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen
SH8934	*ptp2Δ msg5Δ cna2Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 Δcna2::KanMX4 ura3-52* (or *ura3 Δ0*) *his3-Δ200* (or *his3 Δ1*) *leu2Δ1* (*leu2 Δ0*) *lys2Δ202 (lys2 Δ0) trp1Δ63*	(SH6793 x SH8929)-3C
SH8930	*cnb1Δ*	*MAT* α *Δcnb1::KanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen
SH8935	*ptp2Δ msg5Δ cnb1Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 Δcnb1::KanMX4 ura3-52* (or *ura3 Δ0*) *his3-Δ200* (or *his3 Δ1*) *leu2Δ1* (*leu2 Δ0*) *lys2Δ202 (lys2 Δ0) trp1Δ63*	(SH6793 x SH8930)-13A
SH8967	*crz1Δ*	*MAT* α *Δcrz1::KanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen
SH8968	*ptp2Δ msg5Δ crz1Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 Δcrz1::KanMX4 ura3-52* (or *ura3 Δ0*) *his3-Δ200* (or *his3 Δ1*) *leu2Δ1* (*leu2 Δ0*) *lys2Δ202 (lys2 Δ0) trp1Δ63*	(SH6793 x SH8967)-4B

SH8931	*slt2*Δ	*MAT* α *Δslt2::KanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen
SH8972	*ptp2*Δ *msg5*Δ *slt2*Δ	*MAT*〈 *Δptp2*::*CgHIS3 Δmsg5*::*CgLEU2 Δslt2*::*KANMX4 ura3-52* (or *ura3 Δ0*) *his3*-Δ*200* (or *his3 Δ1*) *leu2*Δ*1* (*leu2 Δ0*) *lys2*Δ*202 (lys2 Δ0) trp1*Δ*63*	(SH6793 x SH8931)-2B
SH8986	*ptp2*Δ *msg5*Δ *ste12*Δ	*MAT α Δptp2::CgHIS3 Δmsg5::CgLEU2 Δste12::CgTRP1 ura3-52 his3- Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	Trp[+] disruptant of SH6792 double disruptant using p1805 (*CgTRP1*)
SH8987	*ptp2*Δ *msg5*Δ *sok2*Δ	*MAT α Δptp2::CgHIS3 Δmsg5::CgLEU2 Δsok2::ScURA3 ura3-52 his3- Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	Ura[+] disruptant of SH6792 double disruptant using p3276 (*ScURA3*)
SH8988	*ptp2*Δ *msg5*Δ *ste12*Δ *sok2*Δ	*MAT α Δptp2::CgHIS3 Δmsg5::CgLEU2 Δste12::CgTRP1 Δsok2::ScURA3 ura3-52 his3- Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	Ura[+] disruptant of SH8987 triple disruptant using p3276 (*ScURA3*)

*NBRP/YGRC, National BioResource Project/Yeast Genetic Research Center, Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html)

Table 3. Primers used for disruption and confirmation PCR

Primer name	Primer sequence
Gd PTP2 F	5' ATAACGGCAATAGAATGGCTTCTTCCGCTATATCGGAA AACACAGG AAACAGCTATGACC 3'
Gd PTP2 R	5' GTAGCAATATACTTGAAATCAGGATTAATTTGCGTGAG CTGTTGTAAAACGACGGCCAGT 3'
Co PTP2 F	5' CTCAAGCTTGGAC ACTCGTTTAATTTAGCCA 3'
Co PTP2 R	5' CTCAAGCTTATTCGGTATTGG CACAAACTTT 3'
Gd MSG5 F	5' ACATCGATTTCAAGCCAAACTCACCGCGTTCCTTACAA AACACAGGAAACAGCTATGACC 3'
Gd MSG5 R	5' TCGTTGTCCACAGAAGCTTCCAGTGAATCTGCGGGTTG AGGTTGTAAAACGACGGCCAGT 3'
Co MSG5 F	5' CTCGGATCCGTAGTGATGGATAATGTGATTT 3'
Co MSG5 R	5' CTCGGATCCGTGCCCATGGTAATTTTTGACG 3'
Co CNA1 F	5' CAATAAATACAAACTGTTTG 3'
Co CNA1 R	5' ATTTGAATTTAGAACCGCTT 3'
Co CNA2 F	5' TCTTCTACGTATTTTTGTAT 3'
Co CNA2 R	5' TTCAAAATGGTGAAGTTAGA 3'
Co CNB1 F	5' AATTTGTTTCCCTCGACTTC 3'
Co CNB1 R	5' AGATATCAAAGCTATTAATA 3'
Co CRZ1 F	5' CGTCGAGGACCCTACCCCTG 3'
Co CRZ1 R	5' GAATTGAGCTACTTTTGTCT 3'
Gd STE12 F	5' ATAGCGGAACCGCTTTCTTTATTTGAATTGTCTTGTTC ACCAAGGCACAGGAAACAGCTATGACC 3'
Gd STE12 R	5' CCGCATTTTTAATTCTTGTATCATAAATTCAAAAATT ATATTATAGTTGTAAAACGACGGCCAGT 3'
Co STE12 F	5' ACTCTTCGCGGTCAGGTCTC 3'
Co STE12 R	5' GTCATGTTAAGAAACTCATT 3'
Gd SOK2 F	5' CAGTACAAAATCATCCTTATATAACCCTGGTAAGGT CCTTTTGTCCTTCGTACGCTGCAG 3'
Gd SOK2 R	5' GTTTTGATTAAAGTAACATAATTATCCAAGGAATT CATAGTTGTTGCCACTAGTGGATCT 3'
Co SOK2 F	5' GAAAAAAGTTCATCTTTAAC 3'
Co SOK2 R	5' AGTTTCTCGTTTAAAAAA TC 3'

Table 4. Primers used in RT-PCR

Primer name	Primer sequence
rt ENA1 F	5’ TTGGATCCCTCGCTTTGGT 3’
rt ENA1 R	5’ CAAACTCACGTTGCCCTCATT 3’
rt GSC2 F	5’ CACAGACCTTCACCGCATCTT 3’
rt GSC2 R	5’ GACTCTGCATATTTAGCAGCAAAAAC 3’
rt PMC1 F	5’ GCCATAAGTGTTGCCCACAA 3’
rt PMC1 R	5’ GAGTCTTTATCACGCAGCTGTTCA 3’
rt PMR1 F	5’ ACCACCGCGGCCTGTA 3’
rt PMR1 R	5’ TACCATCTTCGGCCATCTCTTT 3’

2.3 Results

2.3.1 An active calcineurin pathway is inhibitory to the growth of the calcium-exposed *ptp2*Δ*msg5*Δ double disruptant

S. cerevisiae cells generally respond to high extracellular calcium exposure by eliciting the calcium/calmodulin-dependent signaling pathway to maintain cell viability (Cyert, 2003). To determine whether activation of calcineurin occurs in the *ptp2*Δ*msg5*Δ double disruptant upon exposure to calcium, we examined the expression of 120 genes that are known to be regulated by calcineurin using microarray (see Materials and methods). Based on Gene Ontology (GO) categories, 46 out of 102 calcineurin-dependent genes belong to "molecular function" while 23 and 33 out of 102 belong to "cell component" and "biological process", respectively.

Microarray results showed up-regulation of calcineurin-regulated genes in both the *ptp2*Δ*msg5*Δ double disruptant (89.1%) and wild-type (87.7%) strains upon exposure to high extracellular calcium. (Fig. 4, column 1 and column 3, respectively). Interestingly, the expression values corresponding to the up-regulated calcineurin genes in the calcium-exposed *ptp2*Δ*msg5*Δ double disruptant did not vary significantly to that of the calcium-treated wild-type strain although most exhibited slight decrease in expression (Fig.4, column 2).

To confirm these results, the transcription level of several representative genes known to be up-regulated in a calcineurin-dependent manner was validated using real-time RT-PCR (Cunningham and Fink, 1994). These include the ß-1,3-glucan synthase gene *GSC2* (*FKS2*), *PMC1*, *PMR1* and *ENA1*(*PMR2*), in which the latter three encode for P-type ATPases required for ion homeostasis (Yoshimoto et al., 2002). Results of the RT-PCR analysis revealed a similar trend in the expression with the microarray data wherein exposure to calcium caused an increase in the expression level of *GSC2*, *PMC1* and *ENA1* in the

*ptp2*Δ*msg5*Δ double disruptant. Interestingly, expression of *PMR1* was not induced either in the wild type or *ptp2*Δ*msg5*Δdouble disruptant upon exposure to calcium probably because *PMR1* is controlled by other calcium-dependent mechanisms. These results indicate that the calcineurin pathway is active in the *ptp2*Δ*msg5*Δ double disruptant and the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant is not due to a defective calcineurin pathway (Fig. 5).

To strengthen the above conclusion, we studied the localization of Crz1 in the wild type and the *ptp2*Δ*msg5*Δ double disruptant after calcium exposure. Since calcineurin activation is known to cause nuclear localization of Crz1 (Stathopoulos-Genontides et al., 1999), we tagged Crz1 with GFP in both wild type and *ptp2*Δ*msg5*Δ double disruptant. As expected, Crz1 was localized in the nucleus of the calcium-exposed wild type. Similarly, nuclear localization was observed in the *ptp2*Δ*msg5*Δ double disruptant exposed to calcium thereby indicating an active calcineurin pathway in the *ptp2*Δ*msg5*Δ double disruptant upon calcium exposure (Fig. 6).

We inhibited the calcineurin pathway either by FK506 (a calcineurin inhibitor) treatment or disruption of *cnb1*Δ (calcineurin regulatory subunit) to determine the effect of inactivation of the calcineurin pathway on the phenotype of the *ptp2*Δ*msg5*Δ double disruptant. As expected, disruption of either *cna1*Δ or *cna2*Δ in the *ptp2*Δ*msg5*Δ double disruptant did not cause calcium tolerance since *CNA1* and *CNA2* redundantly codes for the calcineurin catalytic unit (Fig. 7B). However, addition of FK506 in the medium (Fig. 7A) or disruption of *CNB1* (Fig. 7B) was able to suppress the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant, indicating that deactivation of the calcineurin pathway in the *ptp2*Δ*msg5*Δ double disruptant can restore growth in a calcium-exposed environment. Taken together, we conclude that transcription of calcineurin genes is not impaired and calcineurin

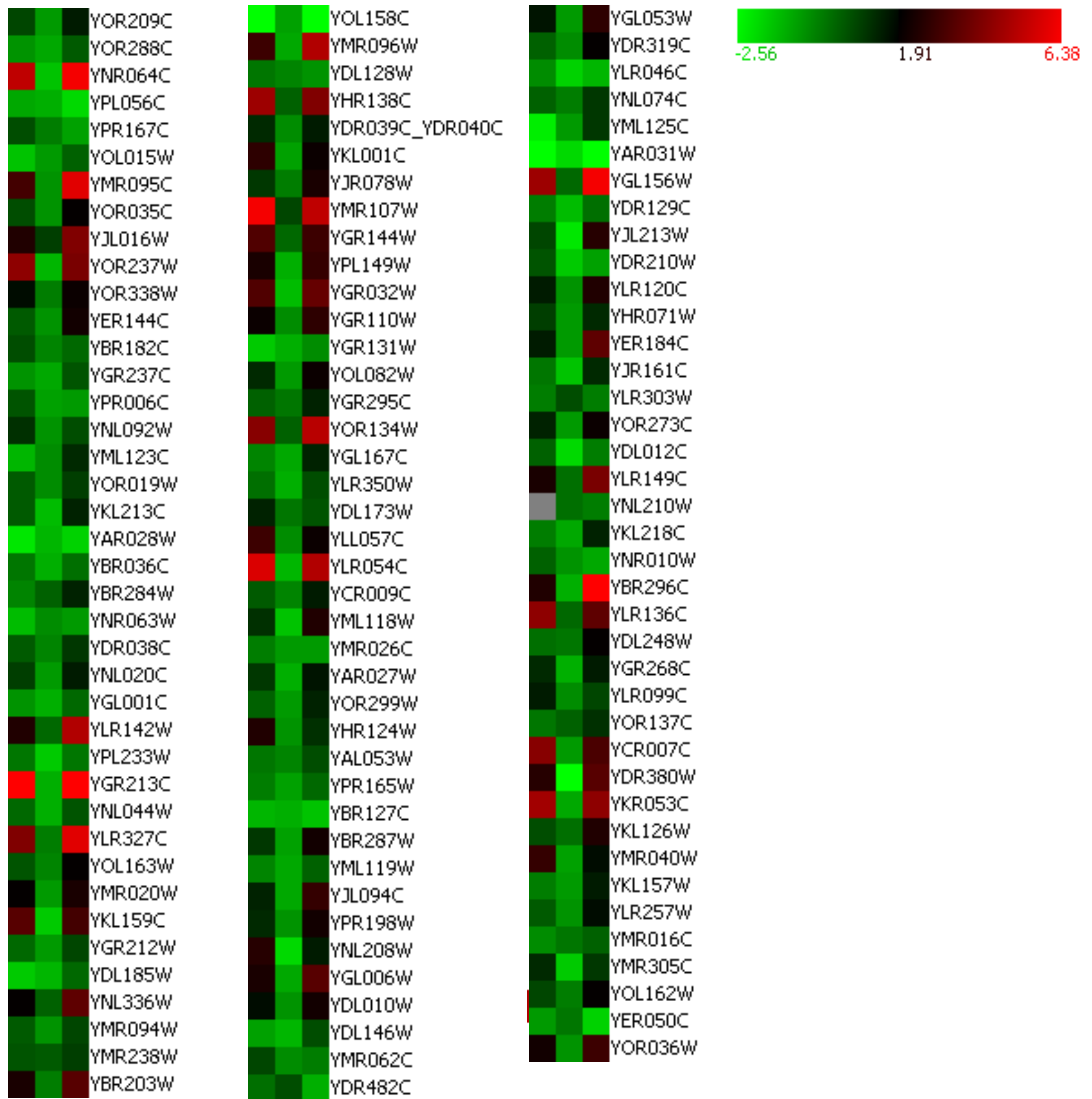

Figure 4. Heatmap of the expression response of calcium regulated-calcineurin genes in the wild type and *ptp2*Δ*msg5*Δ disruptant with or without calcium exposure. The column number indicates the set-up as follows: 1) comparison between Ca^{2+}-exposed *ptp2*Δ*msg5*Δ disruptant with untreated *ptp2*Δ*msg5*Δ disruptant; 2) comparison between Ca^{2+}-exposed *ptp2*Δ*msg5*Δ disruptant with Ca^{2+}-exposed wild type; 3) comparison between Ca^{2+}-exposed wild type with untreated wild type. All strains were cultivated in YPD media and calcium treatment was done by supplementing the YPD medium with 0.3 M $CaCl_2$. Four independent DNA microarray data were analyzed using Genowiz (Ocimum Biosolutions). The data were normalized prior to statistical analysis using the LOWESS algorithm. Genes showing more than two-fold induction correspond to the rows and the columns represent the experimental set-ups. Red and green colors represent up-regulation and down-regulation of expression respectively. The color saturation represents the magnitude of the expression ratio, as indicated by the scale at the right side of the figure.

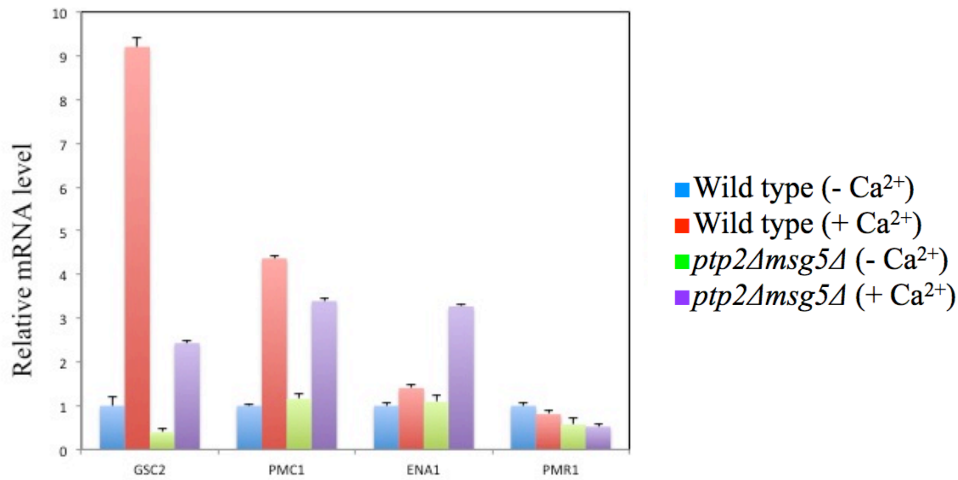

Figure 5. Calcineurin activation occurs in the *ptp2*Δ*msg5*Δ double disruptant. Representative calcineurin genes in the *ptp2*Δ*msg5*Δ double disruptant are up-regulated upon calcium exposure. cDNA synthesized from total RNA isolated from the sample strains grown with (0.3 M $CaCl_2$) or without calcium were used as templates for RT-PCR using SYBR Green and specific primers for *GSC2*, *PMC1, ENA1* and *PMR1*. *ACT1* was used as control. *GSC2, PMC1* and *ENA1* showed increased expression while *PMR1* expression remained the same in the *ptp2*Δ*msg5*Δ double disruptant upon calcium exposure. The results are means of three experiments from three independent preparations.

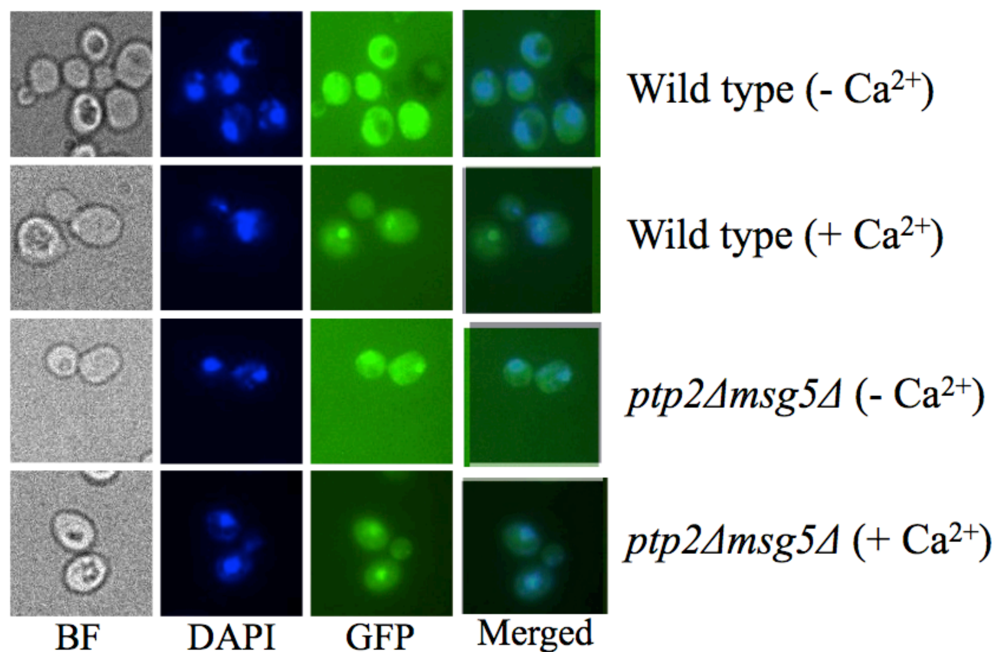

Figure 6. Calcineurin activation occurs in the *ptp2*Δ*msg5*Δ double disruptant. Crz1p localization in the nucleus occurs in the *ptp2*Δ*msg5*Δ double disruptant. Wild type and *ptp2*Δ*msg5*Δ double disruptant cells expressing GFP-tagged Crz1p were grown in medium with or without calcium until mid-log phase. Subcellular localization of GFP-Crz1p (GFP) and DAPI-stained nucleus (DAPI) were observed by fluorescence microscopy. Unstained cells were visualized by bright field microscopy (BF). Approximately 200 individual cells for each strain and treatment were observed for Crz1p localization.

calcineurin activation has a negative effect on the growth of the *ptp2*Δ*msg5*Δ double disruptant upon exposure to calcium.

Interestingly, additional disruption of *CRZ1* in the *ptp2*Δ*msg5*Δ double disruptant background did not confer calcium tolerance. The fact that the *crz1*Δ single disruptant is also calcium sensitive implies that Crz1 is essential to the cell's viability under high extracellular calcium stress conditions. Our result also indicate that *CRZ1* is located downstream of the calcium-induced, growth-related redundant pathways mediated by Ptp2 and Msg5 where the parallel pathways converge (Fig. 7B).

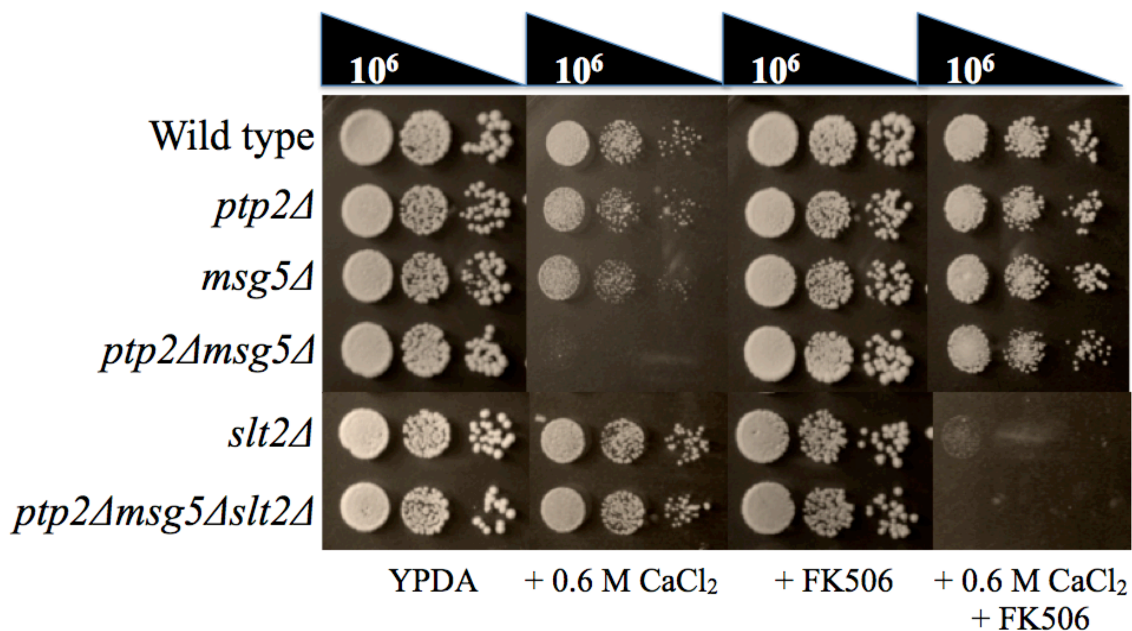

Figure 7A. Inhibition of calcineurin by FK506 treatment suppresses the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant. BY5209 (wild type), ptp2Δ*, msg5*Δ*, slt2*Δ*, ptp2*Δ*msg5*Δ *and ptp2*Δ*msg5*Δ*slt2*Δ were grown in YPD to mid-log phase. Ten-fold serial dilutions of the cell suspensions were prepared and spot plated on to YPDA, YPDA supplemented with 0.6 M $CaCl_2$, YPDA added with FK506 (calcineurin inhibitor) and YPDA with both $CaCl_2$ and FK506. The plates were incubated at 30ºC for 2-4 days.

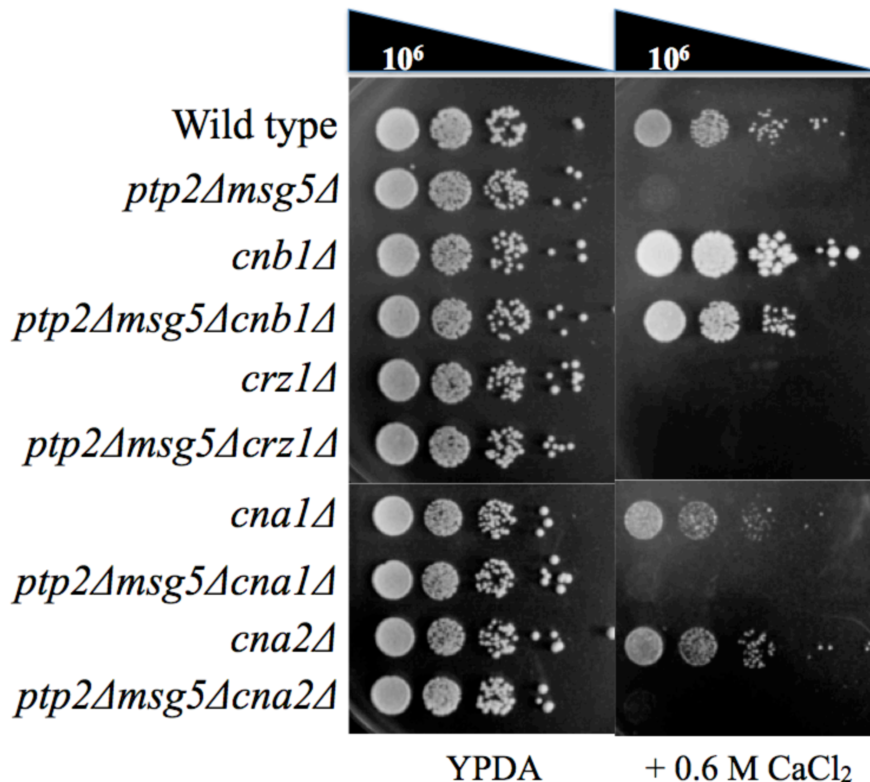

Figure 7B. Inhibition of calcineurin by *CNB1* disruption suppresses the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant. The subunits of calcineurin were individually disrupted in the *ptp2*Δ*msg5*Δ background to produce triple disruptants. *CNA1* and *CNA2* are catalytic units while *CNB1* is the regulatory subunit of calcineurin. BY5209 (wild type), *cna1*Δ, *cna2*Δ, *cnb1*Δ, *crz1*Δ, *ptp2*Δ*msg5*Δ, *ptp2*Δ*msg5*Δ*cna1*Δ, *ptp2*Δ*msg5*Δ*cna2*Δ, *ptp2*Δ*msg5*Δ*cnb1*Δ *and ptp2*Δ*msg5*Δ*crz1*Δ were grown in YPD to mid-log phase. Ten-fold serial dilutions of the cell suspensions were prepared and spot plated on to YPDA with and without 0.6 M $CaCl_2$. The plates were incubated at 30ºC for 2-4 days.

2.3.2 Up-regulated cell wall genes in the *ptp2*Δ*msg5*Δ double disruptant share common transcription factors Sok2 and Ste12

Activation of the SLT2 pathway leads to the induction of transcription factors like Rlm1 and Swi4/Swi6 which in turn stimulates the expression of downstream or effector genes that play roles in the maintenance of cell wall integrity (Watanabe and Matsumoto, 1995; Levin, 2005; Jung and Levin, 1999). Based on this, we inferred that hyper-activation of the SLT2 pathway essentially causes up-regulation of several downstream genes that eventually leads to cell toxicity (Watanabe and Matsumoto, 1995; Hahn and Thiele, 2002). Since many

of the effector genes downstream of the SLT2 pathway are involved in cell wall construction and cell wall-related processes, we used the microarray data to search for cell wall-related genes that are differentially regulated in the calcium exposed-*ptp2*Δ*msg5*Δ double disruptant. Out of the 31 cell wall-related genes that are reported to be differentially expressed during cell cycle, we found five genes namely *CWP1, CHS1, PST1, SCW11* and *EXG1* that are up-regulated while another gene, *CWP2*, was down-regulated in *ptp2*Δ*msg5*Δ double disruptant exposed to calcium (Table 5) (Jung and Levin, 1999; Klis et al., 2006).

Table 5. Cell wall genes that are differentially regulated in the *ptp2*Δ*msg5*Δ double disruptant upon exposure to calcium

Gene name	Molecular function	Log2 expression
CWP1	Structural constituent of cell wall	1.1
CWP2	Structural constituent of cell wall	-1.2
CHS1	Chitin synthase activity	1.2
PST1	Molecular function unknown	1.6
SCW11	Glucan 1,3-beta-glucosidase activity	1.9
EXG1	Glucan 1,3-beta-glucosidase activity	1.1

To find a link between the cell wall genes that are differentially expressed, we looked for transcription factors that were common among the differentially regulated genes. YEASTRACT analysis identified two probable transcription factors shared by the altered genes as Sok2 and Ste12 (Teixeira et al., 2005). However, compared to the wild type, *SOK2* and *STE12* were not differentially expressed in the *ptp2*Δ*msg5*Δ double disruptant; indicating that the mode of regulation involved is post-transcriptional (unpublished data).

To further elucidate the roles of the transcription factors Sok2 and Ste12 in the calcium phenotype of the *ptp2*Δ*msg5*Δ double disruptant, we disrupted *SOK2* and *STE12* independently and compositely in the *ptp2*Δ*msg5*Δ double disruptant background. Results showed that neither disruption of *STE12* or *SOK2* conferred calcium tolerance to the *ptp2*Δ*msg5*Δ double disruptant. However, disruption of both *STE12* and *SOK2* was able to

confer a slightly calcium tolerant phenotype as shown in the quadruple disruptant, *ptp2Δmsg5Δste12Δsok2Δ* (Fig. 8). This indicates that both *STE12* and *SOK2* play roles in the calcium phenotype of the *ptp2Δmsg5Δ* double disruptant and their synergistic relationship negatively affects growth in the presence of calcium.

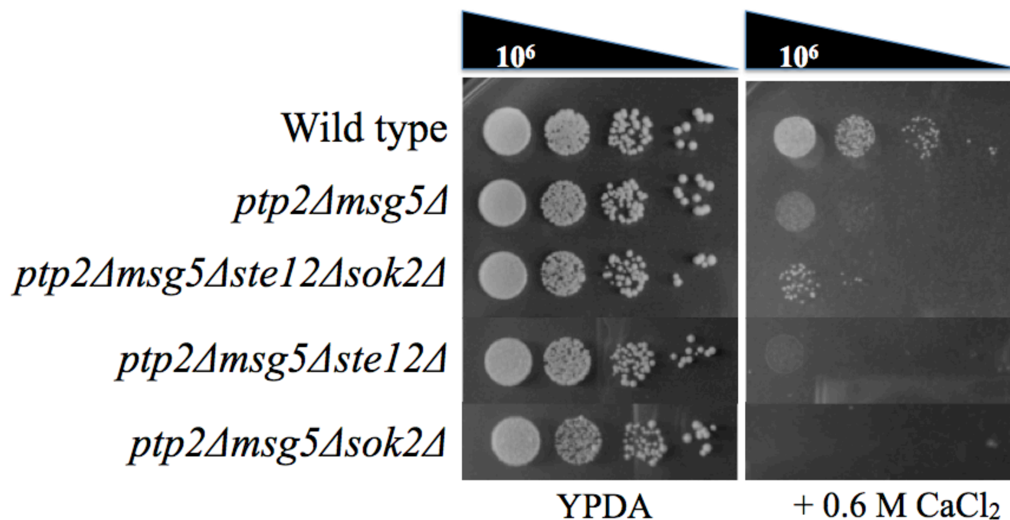

Figure 8. Disruption of both transcription factors Ste12 and Sok2 confers partial calcium tolerance to *ptp2Δmsg5Δ* double disruptant. BY5209 (wild type), *ptp2Δmsg5Δ, ptp2Δmsg5Δste12Δ, ptp2Δmsg5Δsok2Δ and ptp2Δmsg5Δste12Δ sok2Δ* were grown in YPD to mid-log phase. Ten-fold serial dilutions of the cell suspensions were prepared and spot plated on to YPDA with and without 0.6 M $CaCl_2$. The plates were incubated at 30ºC for 2-4 days.

2.3.3 SLT2 pathway is hyper-activated in the *ptp2Δmsg5Δ* double disruptant upon exposure to high extracellular calcium

MAPK signaling cascades are activated by the process of phosphorylation of cascades of protein kinases (Zhao et al., 1998). Furthermore, regulation of these pathways is via dephosphorylation of MAPK by protein phosphatases such as Ptc1, Ptc2, Ptc3, Ptp2, Ptp3 and Msg5 (Martin et al., 2005). Since Ptp2 and Msg5 are known to negatively regulate Slt2 phosphorylation, we confirmed if Slt2 was indeed hyper-phosphorylated in the *ptp2Δmsg5Δ* double disruptant. As expected, Western blot analysis revealed that Slt2 was hyper-phosphorylated in the *ptp2Δmsg5Δ* double disruptant compared to the wild type (Fig. 9). Our

result is consistent with the earlier findings that disruption of both *PTP2* and *MSG5* PPase genes results in a hyper-phosphorylated state of Slt2 (Hermansyah et al., 2009).

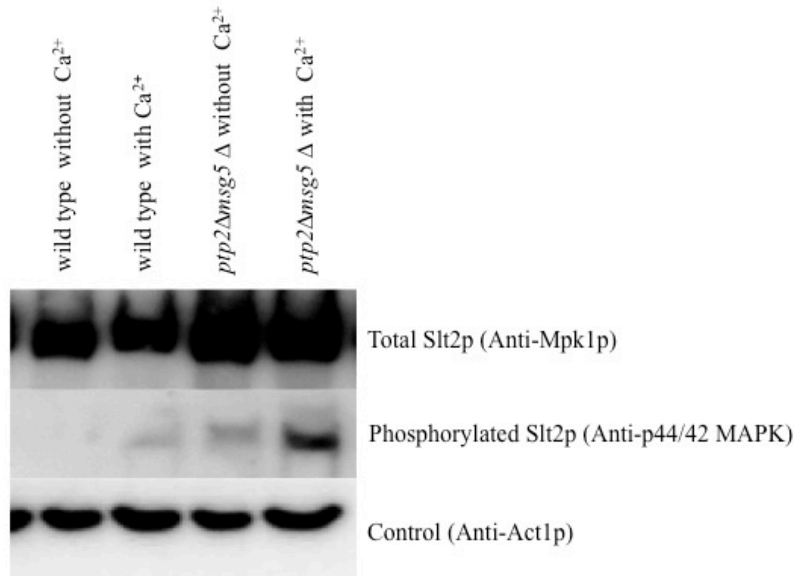

Figure 9. Slt2 is hyper-phosphorylated in the calcium-exposed *ptp2*Δ*msg5*Δ double disruptant. Representative Western blot showing the phosphorylation level of Slt2p. Soluble protein extracts were prepared from cells grown in YPD with (0.3 M $CaCl_2$) or without calcium at mid-log phase. Total Slt2p and the phosphorylated form of Slt2p were detected using anti-Mpk1p (Slt2p) and anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibodies, respectively. Anti-Act1p was used as control.

2.3.4 Calcium sensitivity in the *ptp2*Δ*msg5*Δ double disruptant results from a hyper-activated, calcium-induced signaling pathway caused by the improper activation of the SLT2 pathway and an active calcineurin

We presumed that the simultaneous activation of both SLT2 and calcineurin pathways might be toxic to cells as genetic evidence indicates a functionally redundant relationship between SLT2 and calcineurin pathways in growth-related functions (Nakamura et al., 1996). Thus, we tried to inactivate either one of the redundant pathways (calcineurin or SLT2 pathways) to determine if the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant can be suppressed. Indeed, *slt2*Δ disruption was able to confer calcium tolerance in the *ptp2*Δ*msg5*Δ double disruptant (Hermansyah et al., 2012). Moreover, when calcineurin was inactivated in the *ptp2*Δ*msg5*Δ double disruptant by either disrupting *CNB1* (calcineurin

regulatory subunit gene) or by treatment with a calcineurin inhibitor, FK506, we found that inactivation of calcineurin by either means was also able to suppress the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant (Fig. 7). Thus, calcineurin seems to have an inhibitory role in the growth of the calcium-exposed *ptp2*Δ*msg5*Δ double disruptant under a hyper-phosphorylated SLT2 state (Fig. 7).

Inactivation of both calcineurin (FK506 treatment) and SLT2 pathways (disruption of *slt2*Δ in *ptp2*Δ*msg5*Δ) led to a calcium sensitive phenotype, indicating that the presence of at least one of the redundant pathways is essential for viability under high calcium conditions (Fig. 7A). Taken together, these findings indicate that a hyper-activated signaling state caused by a simultaneously over-activated SLT2 pathway and an active calcineurin pathway led to a calcium sensitive phenotype in the *ptp2*Δ*msg5*Δ double disruptant upon exposure to high extracellular calcium.

2.4 Discussion

We previously reported a PPase double disruptant, *ptp2*Δ*msg5*Δ, exhibiting sensitivity at very high concentrations of calcium (0.6 M). This calcium sensitive phenotype was suppressed by an additional disruption of either one of the six PKases namely *BCK1, MKK1, SLT2, MCK1*, *SSK2* and *YAK1* (Hermansyah et al., 2010). Although we have reported in our earlier study that the SLT2 pathway is over-activated in the *ptp2*Δ*msg5*Δ double disruptant, the detailed mechanism on how a hyper-activated SLT2 pathway causes calcium sensitivity remained unclear. In this work, we clarified the conditions that lead to the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant in terms of functional redundancy at the protein and signal cascade levels.

At the protein level, Ptp2p and Msg5p, which are protein tyrosine (PTP) and dual specificity (DSP) PPases respectively, are known to co-regulate Slt2p (Mpk1p) of the SLT2 (Cell Wall Integrity or Mpk1) pathway (Martin et al., 2005; , Levin, 2005). Based on genetic analysis, we established the redundant roles of these two PPases in the calcium-mediated signaling process since single disruption of either *ptp2*Δ or *msg5*Δ is calcium tolerant (Sakumoto et al., 2002). In general, the MAPK cascade is comprised of a sequential activation of protein kinases. Specifically, MEK kinase (MEKK or MAPKKK) activates the MAPK kinase (MEK or MAPKK) which in turn activates the MAP kinase by dual phosphorylation of its TXY motif (Zhao et al., 2007). This cascade event will eventually result in the activation of various transcription factors or induction of specific sets of genes based on the environmental stimuli. Thus, we presumed that hyper-activation of the SLT2 pathway arises when there is accumulation of hyper-phosphorylated Slt2p in the absence of the negative regulators, Ptp2p and Msg5p (Fig. 10). In this case, a hyper-phosphorylated state of Slt2p is indicative of a hyper-activated SLT2 pathway since the activation of yeast MAPK cascades is through phosphorylation (Zhao et al., 2007). This hyper-activated state of the

SLT2 pathway induces G1 delay by possibly regulating the expression of effector genes such as *CLN2* via several transcription factors as we previously reported (Hermansyah et al., 2009). In connection with this, we reported the down-regulation of *CLN2* in the *ptp2*Δ*msg5*Δ double disruptant upon exposure to high extracellular calcium (Hermansyah et al., 2010). The *CLN2* down-regulation can be explained based on the model for regulation of Swi4 by Slt2 (Mpk1) wherein Slt2 protein is activated by a calcium-induced cell wall stress and is likely to phosphorylate Swi6, causing its exclusion to the nucleus. A non-nuclear localized Swi6 therefore cannot form the SBF complex, resulting in reduction or abolishment of transcription of late G1 genes including *CLN1* and *CLN2* (Levin, 2005).

The fact that the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant exhibits growth arrest (G1 arrest) similar to that of calcineurin-deficient mutants suggested the possible involvement of calcineurin in this signaling pathway. Although essential for survival during stressed conditions, we propose that an active calcineurin coupled with a hyper-activated SLT2 pathway becomes toxic to yeast cells upon exposure to high extracellular calcium (Fig. 7A and 7B). To our knowledge, this is the first report of the existence of a functionally redundant relationship between the SLT2 and calcineurin pathways in response to high levels of calcium. In this case where the SLT2 pathway is hyper-activated due to the absence of the negative regulators Ptp2p and Msg5p, one way to alleviate the harmful effects of an over-active calcium-induced signaling pathway is to inactivate calcineurin by *cnb1*Δ disruption or treatment with a calcineurin inhibitor, FK506 (Fig. 7A and 7B). Therefore, the redundant pathways related to growth serve as safeguards for unwanted effects of an inactive signaling pathway. In this system, a signal cascade assumes the function of another cascade when it is inactivated or impaired. However, improper activation of both signaling cascades becomes harmful to the cells due to signal hyper-activation that eventually leads to cessation of growth. In this study, we have

demonstrated that such is the case for strains lacking signaling cascade regulators such as PPases Ptp2 and Msg5 when exposed to high extracellular calcium (Fig. 8).

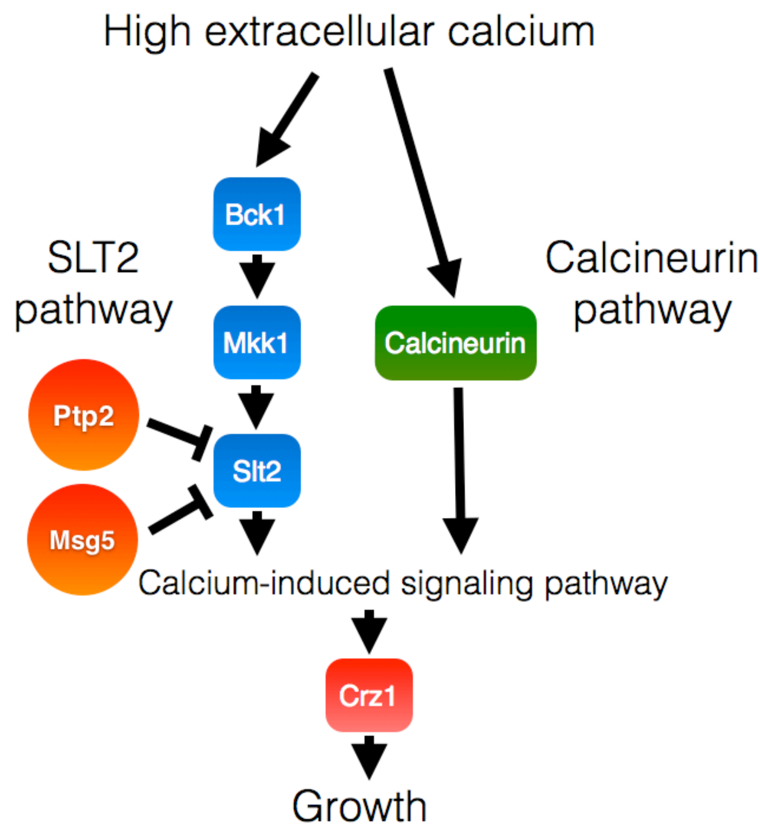

Figure 10. Over-activation of the calcium-induced signaling pathway in the *ptp2Δmsg5Δ* double disruptant causes calcium sensitivity. Calcineurin and SLT2 pathways share redundant functions in cell processes related to growth. Upon exposure to high extracellular calcium, calcineurin is the preferred route while SLT2 pathway is inactivated through dephosphorylation of Slt2p by the functionally redundant Ptp2p and Msg5p. In the absence of both Ptp2 and Msg5 PPases, the SLT2 pathway is hyper-activated and causes growth delay due to a hyper-activated state of the calcium-induced signaling cascade when coupled with an active calcineurin pathway. The two parallel and redundant pathways that are induced by calcium converge at Crz1.

Similar to a published report (Mazur et al., 1995), our result showed that expression of *GSC2* (*FKS2*) is low under normal growth conditions but immediately increases upon exposure to calcium. In the *ptp2Δmsg5Δ* double disruptant exposed to calcium, there was also an increase in *GSC2* expression (2-fold) although at a much lower increment compared to the wild type (6-fold) (data not shown). This can be an indication that the calcineurin pathway is operational but its activity is slightly impaired in the *ptp2Δmsg5Δ* double disruptant. There

was an earlier report of a dual control of *GSC2* expression by PKC and calcineurin pathways induced by heat stress. If calcineurin activity is impaired, it is expected that calcium-induced expression of *GSC2* is also reduced because calcium induction of *GSC2* is not cell integrity pathway-dependent (Zhao et al., 1998). Moreover, *GSC2* expression in response to extracellular calcium is strictly calcineurin-dependent. Our results imply that hyper-activation of the SLT2 pathway possibly has an inhibitory effect on calcineurin activity.

In the advent of sudden exposure to high extracellular calcium, induction of stress response plays an integral role in the cell's survival. It is therefore imperative that *S. cerevisiae* possess parallel, alternative mechanisms to control calcium signaling in cases where the major pathway is impaired (http://oicr.on.ca). However, stringent regulation of these redundant pathways is essential to avoid over-activation. Our results show that the SLT2 and calcineurin pathways co-mediate cellular processes related to growth upon exposure to high extracellular calcium. It is highly probable that upon exposure to calcium, the calcineurin pathway is the primary signaling response that the yeast employs while the SLT2 pathway, being secondary, is only activated in conditions where the calcineurin pathway is completely impaired. In the wild type, this is accomplished by inhibition of the SLT2 pathway via Slt2p dephosphorylation with the functionally redundant PPases, Ptp2 or Msg5 (Fig. 10). However, over-activation of the calcium-induced signaling pathway can occur if both pathways are active and this state promotes growth inhibition. Our results signify that in the event of SLT2 pathway hyper-activation in the presence of high extracellular calcium, calcineurin function can be abrogated to prevent growth inhibition. However, we found that complete inhibition of both pathways by the disruption of *SLT2* pathway genes and treatment of FK506 led to a calcium sensitive phenotype (Fig. 7A), indicating that the presence of at least one of the redundant pathways is essential for viability in high calcium conditions (Fig. 10).

Our results revealed that Crz1 is an essential part of the calcium-induced, growth-related signaling pathway manifested in the *ptp2*Δ*msg5*Δ double disruptant. Genetic analysis suggested that Crz1 is located downstream of the redundant SLT2 and calcineurin pathway and is possibly the point of convergence between the parallel pathways. We also found that common calcineurin and Crz1-induced genes like *PMR1*, *GSC2*, *PMC1* and *ENA1* were not the transcriptional targets of the calcium-induced signaling pathway in the *ptp2*Δ*msg5*Δ double disruptant (Fig. 5), thus pointing out that the parallel pathways might induce the expression of other genes related to growth and stress response. This assumption is reinforced by the fact that representative calcineurin-regulated genes *PMC1* and *ENA1* were induced in the *ptp2*Δ*msg5*Δ*slt2*Δ but not in *ptp2*Δ*msg5*Δ*cnb1*Δ upon exposure to high extracellular calcium conditions (Fig. 11). The identity of the transcriptional targets of the parallel SLT2 and calcineurin pathways mediated by Ptp2 and Msg5 is still unknown.

Interestingly, Sdp1 or Yil113p has been implicated with SLT2 pathway regulation via dephosphorylation of Slt2, which is similar to the function of Ptp2 and Msg5 in the SLT2 pathway (Mattison et al., 1999; Martin et al., 2000; Hahn and Thiele, 2002; Collister et al. , 2002). In the *ptp2Δmsg5Δ* double disruptant, the presence of a functional Sdp1p could not compensate for the absence of both Ptp2 and Msg5, resulting in the over-activation of the calcium-induced signaling pathway and eventually calcium sensitivity. This fact rules out the possibility that Sdp1 is functionally redundant to Ptp2 and Msg5 in relation to the calcium-induced signaling pathway in the *ptp2Δmsg5Δ* double disruptant. In accordance with our previous statement, our unpublished data showed that the *sdp1Δ* single disruptant (BY4742 background) was calcium tolerant (0.6 M $CaCl_2$) which is an indication that Sdp1 activity is stress specific and has no relation to calcium stress.

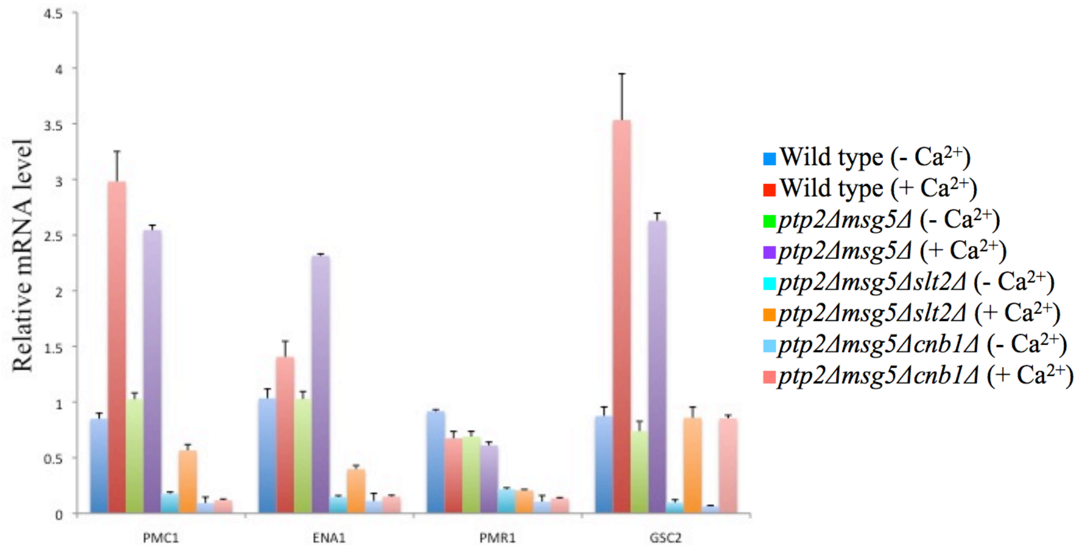

Figure 11. Calcineurin-regulated genes *PMC1* and *ENA1* are induced in the *ptp2*Δ*msg5*Δ*slt2*Δ but not in *ptp2*Δ*msg5*Δ*cnb1*Δ triple disruptant. cDNA synthesized from total RNA isolated from the sample strains grown with (0.3 M $CaCl_2$) or without calcium were used as templates for RT-PCR using SYBR Green and specific primers for *GSC2*, *PMC1, ENA1* and *PMR1*. *ACT1* was used as control. *PMC1* and *ENA1* showed increased expression in the wild type, *ptp2*Δ*msg5*Δ and *ptp2*Δ*msg5*Δ*slt2*Δ but not in *ptp2*Δ*msg5*Δ*cnb1*Δ upon calcium exposure. *PMR1* expression remained the same in all strains upon calcium exposure. *GSC2* showed increased expression in both *ptp2*Δ*msg5*Δ*slt2*Δ and *ptp2*Δ*msg5*Δ*cnb1*Δ, indicating a calcineurin-independent gene induction. The results are means of three experiments from three independent preparations.

This conclusion is supported by reports showing that *YIL113W* disruptant (*sdp1Δ*) exhibited normal response to mating pheromone and has no effect on the phosphorylation of Hog1p MAPK, thus pointing out that Sdp1 is not involved in regulating the response to pheromone and osmotic stress in *S. cerevisiae* (Collister et al., 2002). Results of these studies support the claim that Yil113p phosphatase is a specific regulator of signaling through the Slt2/Mpk1 MAPK pathway and it is highly probable that calcium-induced signaling mediated by the functionally redundant Ptp2 and Msg5 is independent of the Sdp1 function.

This chapter discussed the mechanism of calcium sensitivity in the *ptp2*Δ*msg5*Δ double disruptant and the suppression mechanism conferred by either disrupting SLT2 pathway genes or inactivating calcineurin. The disparity in the mechanism in which calcium tolerance is conferred by *ssk2*Δ or *yak1*Δ disruption remains to be elucidated. Also, the

extent and cause of calcineurin impairment is not presently known and is the subject of future study. In this study, we found proof of the functional redundancy of Ptp2 and Msg5 at the protein level as well as the SLT2 and calcineurin pathways at the cascade level in response to high extracellular calcium conditions. Furthermore, our results imply the important roles of PPases Ptp2 and Msg5 in preventing hyper-activation of SLT2 pathway upon exposure to high calcium concentrations. Lastly, strict modulation of the calcium-induced signaling pathway mediated by the functionally redundant SLT2 and calcineurin pathways is imperative for cell growth under high calcium environments.

Chapter 3

Suppression mechanism of the calcium sensitivity in *Saccharomyces cerevisiae* *ptp2*Δ*msg5*Δ double disruptant involves a novel HOG-independent function of Ssk2, transcription factor Msn2 and the PKA component Bcy1

3.1 Introduction

The growth and survival of eukaryotic cells under stress depend largely on their ability to quickly sense and adapt to the perturbations in the environment. Cells have complex networks of sensing and signaling cascades that are activated appropriately upon specific stress conditions; causing changes in gene expression program, metabolic profile and other cellular features that eventually lead to adaptations of cell growth and proliferation (Hohmann and Mager, 2002). In fact, cells often possess multiple pathways in response to a particular stress thus preventing unwanted consequences of improper activation or inactivation. Previously, we found the existence of functional redundancy in Ptp2 and Msg5 protein phosphatases in response to high extracellular calcium stress (Sakumoto et al., 2002). Suppressor analysis employing protein kinases revealed six suppressors whose additional disruption in the *ptp2*Δ*msg5*Δ double disruptant conferred calcium tolerance namely *BCK1, MKK1* and *SLT2* (Cell Wall Integrity pathway), *MCK1* (chromosome segregation and meiosis), *YAK1* (PKA pathway) and *SSK2* (HOG pathway) (Hermansyah et al., 2010). The suppressor roles of *bck1*Δ*, mkk1*Δ*,* and *slt2*Δ disruptions have been recently explained in Chapter 2 to relate to the co-regulation of calcium signaling in the *ptp2*Δ*msg5*Δ double disruptant by calcineurin and SLT2 pathways that acted as safeguards against sensitivity to high extracellular calcium concentrations (Laviña et al., 2013). On the contrary, FACS analysis implied that *ssk2*Δ and *yak1*Δ suppressors belong to a different regulatory pathway

since they were able to recover from G1 delay unlike *bck1*Δ*, mkk1*Δ*,* and *slt2*Δ suppressors (Hermansyah et al., 2010).

Ssk2 is the upstream MAP kinase kinase kinase of the HOG pathway that phosphorylates Pbs2 after its interaction with Ssk1 (Hohmann, 2002). Pbs2, in turn, phosphorylates and activates the MAP kinase Hog1 that results in the expression of genes involved in glycerol synthesis (Gustin et al, 1998). SSK2 has a close homolog, SSK22, which is redundant with Ssk2 in terms of Pbs2 phosphorylation (Maeda et al., 1995; Posas and Saito, 1998). In addition, Ssk2 has a specialized function in facilitating actin cytoskeleton reassembly after osmotic stress and at the end of the cell cycle (Yuzyuk et al., 2002). These specific functions of Ssk2 have been reported to be unrelated to other known components of the HOG pathway.

Yak1 is a serine-threonine kinase that acts as an antagonist for cell growth (Garrett et al., 1991). Yak1 can be directly phosphorylated and indirectly activated transcriptionally by PKA indicating that Yak1 is downstream of PKA (Pratt et al., 2007). The current model states that an active PKA represses the activity of transcription factors Msn2 and Msn4, thereby leading to the down-regulation of STRE genes that include *YAK1* (Kassis et al., 2000).

In this chapter, we describe a HOG-independent function of Ssk2 that mediates the calcium-sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant. Also, we report new suppressors, *msn2*Δ and *bcy1*Δ*,* which are possibly related to the suppression mechanism conferred by *ssk2*Δ disruption.

3.2 Materials and methods

3.2.1 Strains, media and general methods

S. *cerevisiae* strains used in this study are summarized in Table 6. FY833 strain (SH5209) (*MAT*a *ura3-52 his3-Δ200 leu2Δ1 lys2Δ202 trp1Δ63*) was used as the wild type strain. Yeast strains were grown at 30°C in standard rich medium (YPD) (Sigma-Aldrich), SC medium and SPM medium as described previously (Amberg et al., 2005). YPDA was supplemented with 0.6 M $CaCl_2$ for the calcium phenotype test while 0.3 M $CaCl_2$ was used for mRNA and protein isolation. *Escherichia coli* strains used as sources of plasmids including BYP1804 for p1804 (*CgHIS3*), BYP1805 for p1805 (*CgTRP1*), BYP1419 for p1807 (*CgLEU2*), SHB3276 for p3276 (*ScURA3*) were acquired from NBRP, YGRC, Japan and cultivated in LB medium containing 100 ⌠g/ml ampicillin at 37°C.

Single, double and triple disruptants were constructed using PCR-mediated gene disruption method or mating as previously described (Amberg et al., 2005; Gietz and Schiestl, 2007; Sherman and Hicks, 1991). Confirmation PCR using specific primers was done to verify the correct disruption of genes. The complete list of primers used in the construction and confirmation of the different kinds of disruptants is described on the Table 7.

For spot assay, yeast strains were grown on YPD broth until mid-logarithmic phase, after which the cell number was determined using a spectrophotometer (Smartspec™300, Biorad). The cell suspension was diluted ten-fold using sterile water in a 96-well plate and spotted on appropriate media (YPDA, YPDA with 0.6M $CaCl_2$ or SC medium).

3.2.2 Protein isolation and immunoblot analysis

Protein extracts for Western blot were prepared using the trichloroacetic acid (TCA) method from cells grown until mid-log phase (OD_{660} = 1.0) at 30°C in YPD medium with or

without 0.3M $CaCl_2$ (An et al., 2006). The collected supernatant was used for western blotting. Protein extracts fractionated by SDS-PAGE using 10% polyacrylamide gels were transferred to PVDF Immobilon transfer membranes (Millipore). Total Hog1 was detected with anti-Hog1 (yC-15) antibody (Santa Cruz Biotechnology) while phosphorylated Hog1 was detected with anti-phospho-p38 MAP Kinase (Thr^{180}/Tyr^{182}) antibody (Cell Signaling Technology). Immunoblots were developed with horseradish peroxidase-conjugated anti-goat secondary antibody for total Hog1 and horseradish peroxidase-conjugated anti-rabbit secondary antibody for phosphorylated Hog1using ECL, western lightning™ chemiluminescence (PerkinElmer).

3.2.3 RNA isolation and microarray analysis

RNA was isolated using the hot phenol method with some modifications as described previously (Laviña et al., 2013). Preparation of the cDNA targets, hybridization and washing of the DNA microarray, fluorescence intensity measurement and gene expression analyses were all carried out following the manufacturer's protocol (Ambion Inc., TX, USA) (Laviña et al., 2013). The microarray-based global gene expression analysis of the *ptp2⊗msg5⊗* double disruptant was performed in triplicates.

3.2.4 Gene enrichment analysis using Genowiz

Microarray data were analyzed for distinctive expression using Genowiz™ 4.0 (Ocimum Biosolutions, India). Replicated gene values were combined and mean values of the expression ratios were considered for the dataset while empty spots were removed by filtering. The data were normalized prior to statistical analysis using the LOWESS algorithm (Yang et al., 2002). To stabilize the variation in the dataset, log transformation (log2) and mean centering were performed to bring down the data distribution of dataset near to zero.

Fold change analysis was done for detection of highly expressed genes in which genes with 2 folds up-regulation or down-regulation were considered as differentially expressed. ANOVA and Student's t-test were applied on the data to further establish the highly expressed genes with high degree of confidence, where p-value < 0.05 was considered significant in all statistical analyses. Filtering for the highly fluctuated genes was conducted using the FOREST algorithm (Leman et al., 2007). Two unsupervised methods of clustering namely K-means and Ward's agglomeration method were employed to group the highly fluctuated genes into different clusters based on the gene expression similarity. Functional classification of the genes was performed using Gene Ontology (GO) and pathway analysis using the web-based Gene Ontology (GO) enrichment analysis tool GOstat (http://gostat.wehi.edu.au). Prediction of protein-protein interactions was done using the web based STRING 9.0 resource (http://string-db.org).

3.2.5 Determination of mRNA level using quantitative PCR

Total RNA was isolated from yeast cells grown in YPD broth with or without calcium until mid-logarithmic phase using hot phenol extraction. First strand cDNA synthesis was done using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and the synthesized cDNA was used as the template for real-time RT-PCR. Quantitative PCR was performed using 20 µl reaction mixtures on an Applied Biosystems 7300 Real-Time PCR System with SYBR® Green PCR Master Mix (Applied Biosystems). PCR was performed using the following amplification program: initial denaturation for 10 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C and annealing for 1 min at 60°C. *ACT1* was used as the reference gene for normalization of mRNA levels. The RT-PCR primers used in this study were designed using Primer Express version 2.0 (Applied Biosystems) and are described on the Table 8.

Table 6. *S. cerevisiae* strains used in the study

Strains	**Alias**	**Genotype**	**Source**
SH6314	BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen
SH5209	BY5209 or FY833	*MATa ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63*	NBRP, YGRC*
SH5210	BY5210 or FY834	*MATα ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63*	NBRP, YGRC*
SH6790	*ptp2Δ*	*MATa ptp2Δ::CgHIS3 ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63*	SH5209 disruptant
SH6791	*msg5Δ*	*MATα msg5Δ::CgLEU2 ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63*	SH5210 disruptant
SH6792	*ptp2Δmsg5Δ*	*MATα ptp2Δ ::CgHIS3 msg5Δ::CgLEU2 ura3-52 his3Δ 200 leu2Δ 1 lys2Δ 202 trp1Δ 63*	Meiotic segregant 1B from SH6790 x SH6791
SH6793	*ptp2Δmsg5Δ*	*MATa ptp2Δ::CgHIS3 msg5Δ::CgLEU2 ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63*	Meiotic segregant 2B from SH6790 x SH6791
SH8991	*ptp2Δmsg5Δ ssk2Δ*	*MATα ptp2Δ ::CgHIS3 msg5Δ ::CgLEU2 ssk2Δ ::CgTRP1 ura3-52 his3Δ 200 leu2Δ 1 lys2Δ 202 trp1Δ 63*	Trp^{+} disruptant of SH6792 double disruptant using p1805 (*CgTRP1*)
SH8992	*ptp2Δmsg5Δ ssk22Δ*	*MATα ptp2Δ ::CgHIS3 msg5Δ ::CgLEU2 ssk22Δ ::CgTRP1 ura3-52 his3Δ 200 leu2Δ 1 lys2Δ 202 trp1Δ 63*	Trp^{+} disruptant of SH6792 double disruptant using p1805 (*CgTRP1*)
SH8993	*ptp2Δmsg5Δ pbs2Δ*	*MATα ptp2Δ ::CgHIS3 msg5Δ ::CgLEU2 pbs2Δ ::CgTRP1 ura3-52 his3Δ 200 leu2Δ 1 lys2Δ 202 trp1Δ 63*	Trp^{+} disruptant of SH6792 double disruptant using p1805 (*CgTRP1*)
SH8994	*ptp2Δmsg5Δ hog1Δ*	*MATα ptp2Δ::CgHIS3 msg5Δ ::CgLEU2 hog1Δ ::CgTRP1 ura3-52 his3Δ 200 leu2Δ 1 lys2Δ 202 trp1Δ 63*	Trp^{+} disruptant of SH6792 double disruptant using p1805 (*CgTRP1*)
SH8995	*ptp2Δmsg5Δ bcy1Δ*	*MATα ptp2Δ ::CgHIS3 msg5Δ ::CgLEU2 bcy1Δ ::CgTRP1 ura3-52 his3Δ 200 leu2Δ 1 lys2Δ 202 trp1Δ 63*	Trp^{+} disruptant of SH6792 double disruptant using p1805 (*CgTRP1*)
SH8996	*ptp2Δmsg5Δ msn2Δ*	*MATα ptp2Δ ::CgHIS3 msg5Δ ::CgLEU2 msn2Δ ::loxP-ScURA3-loxP ura3-52 his3Δ 200 leu2Δ 1 lys2Δ 202 trp1Δ 63*	Ura^{+} disruptant of SH6792 double disruptant using p3276 (*ScURA3*)
SH8997	*msn4Δ*	*MATa msn4Δ::loxP-CgTRP1-loxP ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63*	Trp^{+} disruptant of BY5209 using p3010 (*CgTPR1*)
SH8998	*ptp2Δmsg5Δ msn4Δ*	*MATα ptp2Δ ::CgHIS3 msg5Δ ::CgLEU2 msn4Δ ::loxP-CgTRP1-loxP ura3-52 his3Δ 200 leu2Δ 1 lys2Δ 202 trp1Δ 63*	Meiotic segregant 3C from SH8996 x SH8997
SH8999	*ptp2Δmsg5Δ msn2Δmsn4Δ*	*MATα ptp2Δ ::CgHIS3 msg5Δ ::CgLEU2 msn2Δ ::loxP-ScURA3-loxP msn4Δ ::loxP-CgTRP1-loxP ura3-52 his3Δ 200 leu2Δ 1 lys2Δ 202 trp1Δ 63*	Meiotic segregant 4D from SH8996 x SH8997

Table 7. Primers used for gene disruption and confirmation

Primer name	Primer sequence
Kf-PTP2	5’ ATAACGGCAATAGAATGGCTTCTTCCGCTATATCGGAAAACAC AGGAAACAGCTATGACC 3’
Kr-PTP2	5’ GTAGCAATATACTTGAAATCAGGATTAATTTGCGTGAGCTGTT GTAAAACGACGGCCAGT 3’
Kfc-PTP2	5’ CTCAAGCTTGGACACTCGTTTAATTTAGCCA 3’
Krc-PTP2	5’ CTCAAGCTTATTCGGTATTGGCACAAACTTT 3’
Kf-MSG5	5’ ACATCGATTTCAAGCCAAACTCACCGCGTTCCTTACAAAAC ACAGGAAACAGCTATGACC 3’
Kr-MSG5	5’ TCGTTGTCCACAGAAGCTTCCAGTGAATCTGCGGGTTGAG GTTGTAAAACGACGGCCAGT 3’
Kfc-MSG5	5’ CTCGGATCCGTAGTGATGGATAATGTGATTT 3’
Krc-MSG5	5’ CTCGGATCCGTGCCCATGGTAATTTTTGACG 3’
gd SSK2	5’ AAAAGAAGAGAAGCCTTTGCGTAAACTATTTGACAGGCAC AAATACTTCGTACGCTGCAG 3’
gd SSK2	5’ AAAAGAAGAGAAGCCTTTGCGTAAACTATTTGACAGGCAC AAATACTTCGTACGCTGCAG 3’
co SSK2F	5’ TAGAAAGAAGCCAAATCTGC 3’
co SSK2R	5’ TGTTAAAAGCGATGTCTTCT 3’
gd SSK22F	5’ ACTTAGGGTGGCTATAAAAGGTAGTTCCTTGTAGGTGAAA CACAGGAAACAGCTATGACC 3’
gd SSK22R	5’ TATATATCGTAGTATATCATATTTTTAGACGTTGACCACT GTTGTAAAACGACGGCCAGT 3’
co SSK22F	5’ GAAATTTGTTAGGAAAACCC 3’
co SSK22R	5’ CTTCTCTGGGAAGTTGAGCC 3’
gd PBS2	5’ ATTATTATATTAAGCAGATCGAGACGTTAATTTCTCAAAG CACAGGAAACAGCTATGACC 3’
gd PBS2	5’ TATATTCACGTGCCTGTTTGCTTTTATTTGGATATTAACG GTTGTAAAACGACGGCCAGT 3’
co PBS2F	5’ TGTCTACTAGTGAGCGATTT 3’
co PBS2R	5’ ACACAATATATTGACGTCCA 3’
gd HOG1F	5’ AAAGGGAAAACAGGGAAAACTACAACTATCGTATATAATA CACAGGAAACAGCTATGACC 3’
gd HOG1R	5’ GAAGTAAGAATGAGTGGTTAGGGACATTAAAAAAACACGT GTTGTAAAACGACGGCCAGT 3’
co HOG1F	5’ GTCTGGCGGCGTTATTATAC 3’
co HOG1 R	5’ AGATAGAAGTGCTGATAAAC 3’
gd BCY11F	5’ TACAACAAGCAGATTATTTTCAAAAGACAACAGTAAGAATAA ACGCTTCGTACGCTGCAG 3’
gd BCY1R	5’ GAGAAAGGAAATTCATGTGGATTTAAGATCGCTTCCCCTTTT TACGCCACTAGTGGATCT 3’
co BCY1F	5’ ATAAGCTGAACTTATTGCAT 3’
co BCY1R	5’ TTTTTGTATGTCACTTGATT 3’

gd MSN2F	5’ TTTCTTTTTCAACTTTTATTGCTCATAGAAGAACTAGATCT AAACTTCGTACGCTGCAG 3’
gd MSN2R	5’ TTATCTTATGAAGAAAGATCTATCGAATTAAAAAAATGGGGT CTAGCCACTAGTGGATCT 3’
co MSN2F	5’ CGGGAAGATCACAACAGTAG 3’
co MSN2R	5’ ACCCCTCTTGCTTTTGTACG 3’
gd MSN4F	5’ ATCAGTTCGGCTTTTTTTTCTTTTCTTCTTATTAAAAACAAT ATACTTCGTACGCTGCAG 3’
gd MSN4R	5’ TACCGTAGCTTGTCTTGCTTTTATTTGCTTTTGACCTTATTT TTTGCCACTAGTGGATCT 3’
co MSN4F	5’ AACCCGAGCTAGAACTAGGA 3’
co MSN4R	5’ CATACCGTAGCTTGTCTTGC 3’

Table 8. RT-PCR primers used in the study

Primer name	Sequence
rt GRE1F	5’ TGCTCAAAGTAACCGCTACCAA 3’
rt GRE1R	5’ TCGTTTCCTGACCCAGACAGA 3’
rt HSP12F	5’ CCGAAAAAGGCAAGGATAACG 3’
rt HSP12R	5’ CGGCTCCCATGTAATCTCTAGCT 3’
rt HSP26F	5’ TGGGTGAAGGCGGCTTAA 3’
rt HSP26R	5’ AGAATCCTTTGCGGGTGTGT 3’
rt NCA3F	5’ CCCTTTCTTCTGTCGCATTTTC 3’
rt NCA3R	5’ TCATCTTTGTGATGATCTTCATGGT 3’
rt PDC6F	5’ GATGGGCTGGTAATGCAAATG 3’
rt PDC6R	5’ CCTTGATGCGTGCGTAACC 3’
rt SIP18F	5’ GGAATGAAGATGGGCCATGA 3’
rt SIP18R	5’ TCCAATCGTTCGCAATTCCT 3’
rt SPS100F	5’ CGAATTTTACGAGCGCACAA 3’
rt SPS100R	5’ TGGACTGGAGGATGATGAGGAT 3’
rt TKL2F	5’ CGATTTGACACCTTCGAATCTG 3’
rt TKL2R	5’ TGGGTAATGGGAGGTTGGAA 3’
rt ALD3	5’ TGGGTGAAGGCGGCTTAA 3’
rt ALD3	5’ TCCTTTGCGGGTGTGTTTG 3’
rt NQM1F	5’ AATGCTGTTGGTATGGCAATAGC 3’
rt NQM1R	5’ GGGAAAGCCATCCTCGTTATAA 3’

3.3 Results

3.3.1 The *PTP2* and *MSG5*-mediated calcium response involves a novel function for Ssk2, a protein kinase component of HOG pathway

Previously, we presumed that HOG pathway is involved in the mechanism mediating the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant since additional *ssk2*⊗ disruption acts as a suppressor of the phenotype (Hermansyah et al., 2010). To clarify the nature of HOG pathway's involvement, we checked the phosphorylation of Hog1 in the wild type, *ptp2Δmsg5Δ* double disruptant and *ptp2Δmsg5Δssk2Δ* triple disruptant. Western blot analysis using anti-phospho-Hog1p demonstrated that exposure to calcium caused an increase in the phosphorylated Hog1 level in the wild type and *ptp2Δmsg5Δ* double disruptant with the phosphorylated Hog1 level being higher in the calcium-exposed *ptp2Δmsg5Δ* double disruptant than in the wild-type strain. Interestingly, there was a decrease in the phosphorylated Hog1 in the *ptp2Δmsg5Δssk2Δ* triple disruptant upon exposure to calcium, which indicated that Hog1 phosphorylation is negatively affected by an additional *ssk2*⊗ disruption in the *ptp2Δmsg5Δ* background. (Fig.12A). Next, we disrupted the other protein kinase components of the HOG cascade in the *ptp2Δmsg5Δ*. In the case of the *ssk2Δ* suppressor, we determined if other elements of the HOG pathway are also involved in the same suppression mechanism since *SSK2* and *PTP2* are components of the HOG pathway as MAPKKK and negative regulator, respectively (Wurgler-Murphy et al., 1997; Tatebayashi et al., 2003). Results showed that additional disruption of *SSK22, PBS2* or *HOG1* in the *ptp2Δmsg5Δ* background did not confer calcium tolerance to the double disruptant (Fig. 12B). Our result signifies that the HOG pathway is not directly involved in the suppression mechanism of the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant. Thus, we

hypothesize that *SSK2* has a novel function in the calcium stress pathway that is independent of its putative function in the HOG pathway.

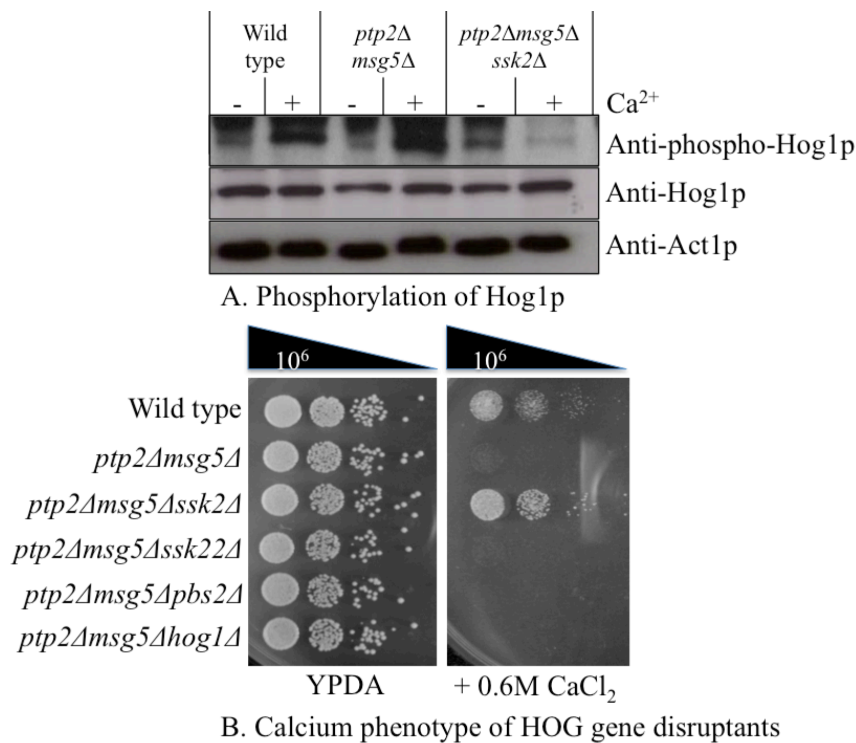

Figure 12. *ssk2*Δ disruption suppresses the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant via a HOG pathway-independent manner. A) Western blot analysis of the wild type, *ptp2*Δ*msg5*Δ double disruptant and *ptp2*Δ*msg5*Δ*ssk2*Δ triple disruptant using anti-Hog1 (yC-15) antibody (Santa Cruz Biotechnology) for total Hog1 and anti-phospho-p38 MAP Kinase (Thr^{180}/Tyr^{182}) antibody (Cell Signaling Technology) for the phosphorylated Hog1. B) Additional *ssk2*Δ disruption confers calcium tolerance to the *ptp2*Δ*msg5*Δ double disruptant. Triple disruptants were constructed by an additional disruption of a HOG pathway kinase in a *ptp2*Δ*msg5*Δ background. Other protein kinase components of the HOG pathway did not confer calcium tolerance to the *ptp2*Δ*msg5*Δ double disruptant.

3.3.2 19 induced genes in the calcium sensitive *ptp2*Δ*msg5*Δ double disruptant are repressed in the calcium tolerant *ptp2*Δ*msg5*Δ*ssk2*Δ triple disruptant

Using microarray analysis, we also attempted to determine genes that might be involved in the suppression mechanism conferred by *ssk2*Δ disruption to the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant by searching for genes with altered expression in the *ptp2*Δ*msg5*Δ and *ptp2*Δ*msg5ssk2*Δ background compared to the wild type. From the initial chip set used, 5,725 usable gene spots were chosen for variance stabilization

across seven experimental grouped samples based on the effects of calcium exposure and gene disruption (*ptp2*Δ, *msg5*Δ and *ssk2*Δ) described in Fig 13. From the list of 42 genes that showed significant fluctuation in gene expression (confidence limit = 0.05), 19 genes with distinct "rise and fall" pattern of expression were selected for further analysis (Fig. 13, Table 9). More specifically, "rise and fall" patterned expression refers to induction of genes in the calcium-sensitive *ptp2*Δ*msg5*Δ double disruptant strains in comparison to the calcium-tolerant wild type following exposure to high extracellular calcium (Fig. 13, columns 3, 6 and 7) and subsequent repression of the same set of genes in the calcium-tolerant *ptp2*Δ*msg5*Δ*ssk2*Δ triple disruptant (Fig. 13, column 1). The genes were selected based on the hypothesis that the gene expression pattern directly correlates with the calcium phenotype of the strains in which increased expression of a set of genes is responsible for the calcium sensitive phenotype and that down-regulation of the same genes confers calcium tolerance.

To confirm the validity of the "rise and fall" patterned expression, we selected the top 10 genes with the highest fluctuation (Standard Deviation) values and determined their expression by real time RT-PCR (Table 9). Results showed the characteristic "rise and fall" pattern in all the genes tested thereby confirming the up-regulation and down-regulation of the genes in the *ptp2*Δ*msg5*Δ and *ptp2*Δ*msg5*Δ*ssk2*Δ*,* respectively (Fig. 14). Taken together, we infer that the "rise and fall" pattern in gene expression is related to the calcium phenotype of the strains and their down-regulation may play a role in the suppression of the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant.

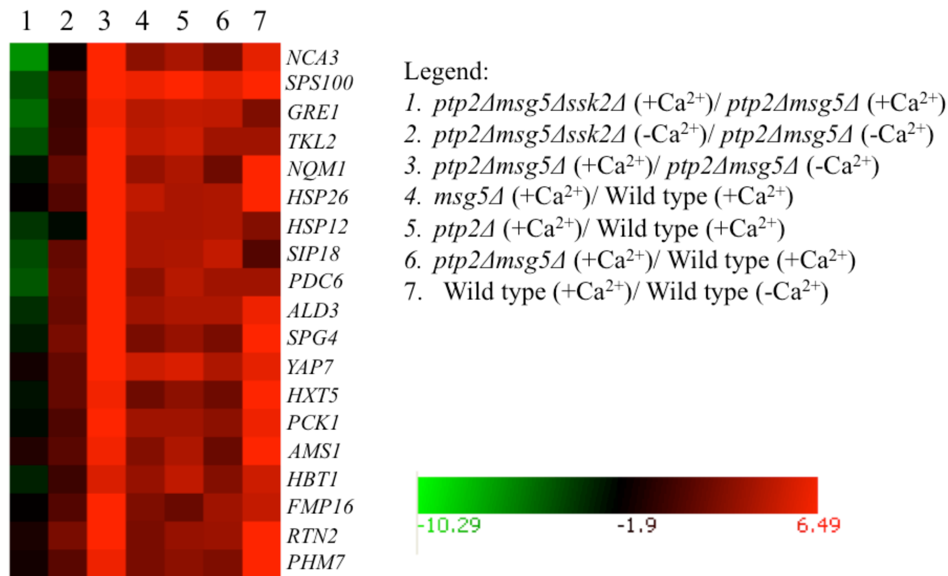

Figure 13. Heat map showing 19 highly fluctuated genes with significant ”rise and fall” patterned expression (3-fold). A) Different experimental set-ups are shown in columns and are listed as follows: 1) calcium-exposed *ptp2Δmsg5Δssk2Δ* triple disruptant and calcium-exposed *ptp2Δmsg5Δ* double disruptant; 2) untreated *ptp2Δmsg5Δssk2Δ* triple disruptant and untreated *ptp2Δmsg5Δ* double disruptant; 3) calcium-exposed *ptp2Δmsg5Δ* double disruptant and untreated *ptp2Δmsg5Δ* double disruptant; 4) calcium-exposed *msg5Δ* single disruptant and calcium-exposed wild type; 5) calcium-exposed *ptp2Δ* single disruptant and calcium-exposed wild type; 6) calcium-exposed *ptp2Δmsg5Δ* double disruptant and calcium-exposed wild type; and 7) calcium-exposed wild type and untreated wild type. The group was determined by k-clustering with the genes arranged based on standard deviation values (SD) with the highest value on the top.

The genes were then analyzed for predicted and known interactions using the STRING web resource (http://string-dg.org) to obtain hints on the overall perspective of the interacting genes or proteins (Szklarczyk et al., 2010). All genes with the exception of *PCK1* and *YAP7* showed linkage in the STRING analysis of the “rise and fall” genes implying the importance of their interaction in the calcium signaling pathway mediated by Ptp2 and Msg5 (Fig. 15). Also, the hierarchical relationships (Gene Ontology) of the “rise and fall” gene cluster were determined using GOstat (http://gostat.wehi.edu.au) in which some genes including *GRE1, HSP12, ALD3, HSP26* and *SIP18* were over-represented in the “response to stress” category (p = 0.000177).

Table 9. Highly fluctuated genes exhibiting "rise and fall" pattern in the *ptp2Δmsg5Δ* and *ptp2Δmsg5Δssk2Δ* disruptants compared to the wild-type strain (confidence limit = 0.05)

Systematic name	Standard name	Function	SD
YJL116C	*NCA3*	Regulation of mitochondrial expression of subunits the Fo-F1 ATP synthases	3.55
YHR139C	*SPS100*	Spore wall maturation	3.50
YPL223C	*GRE1*	Hydrophilin for dessication-rehydration process	3.07
YBR117C	*TKL2*	Transketolase	3.01
YGR043C	*NQM1*	Transalsolase	3.00
YBR072W	*HSP26*	Small heat shock protein with chaperone activity	2.82
YFL014W	*HSP12*	Involved in maintaining membrane organization in stress conditions	2.80
YMR175W	*SIP18*	Essential to desiccation-rehydration process	2.80
YGR087C	*PDC6*	Involved in amino acid catabolism	2.78
YMR169C	*ALD3*	Involved in beta-alanine synthesis	2.72
YMR107W	*SPG4*	Protein required for survival at high temperature during stationary phase	2.64
YOL028C	*YAP7*	Transcription factor	2.44
YHR096C	*HXT5*	Hexose transporter	2.43
YKR097W	*PCK1*	Key enzyme in gluconeogenesis	2.35
YGL156W	*AMS1*	Vacuolar alpha mannosidase	2.32
YDL223C	*HBT1*	Polarized cell morphogenesis	2.30
YDR070C	*FMP16*	Unknown	2.30
YDL204W	*RTN2*	Stabilizes membrane curvature	2.19
YOL084W	*PHM7*	Unknown	2.17

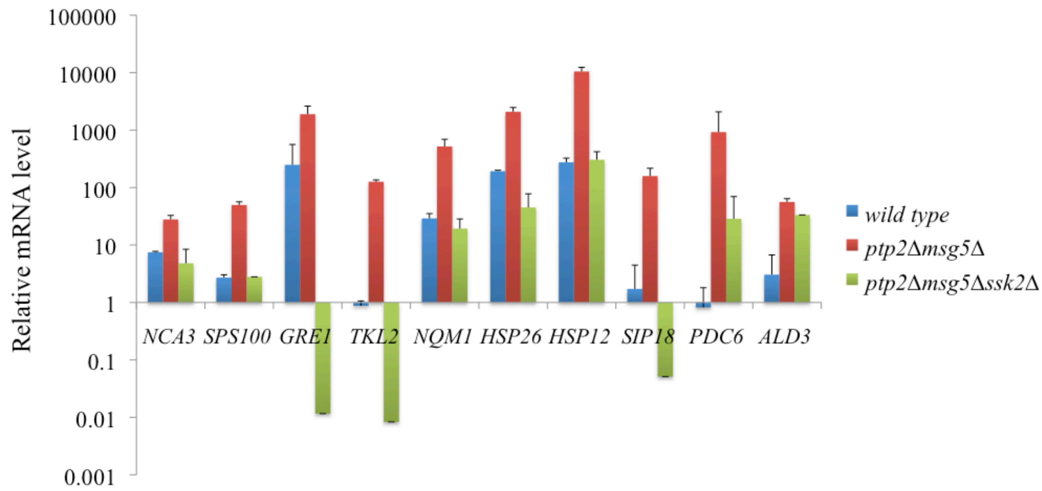

Figure 14. Representative genes showing "rise and fall" patterned expression in the *ptp2Δmsg5Δ* double disruptant and *ptp2Δmsg5Δssk2Δ* triple disruptant as compared to the wild type. cDNA synthesized from total RNA isolated from the sample strains grown with (0.3 M $CaCl_2$) or without calcium were used as templates for RT-PCR using SYBR Green and specific primers for each gene. *ACT1* was used as an internal control. A "rise and fall" pattern designates the up-regulation of genes in the *ptp2Δmsg5Δ* double disruptant and subsequent down-regulation in the *ptp2Δmsg5Δssk2Δ* triple disruptant. The results are means of three experiments from three independent preparations.

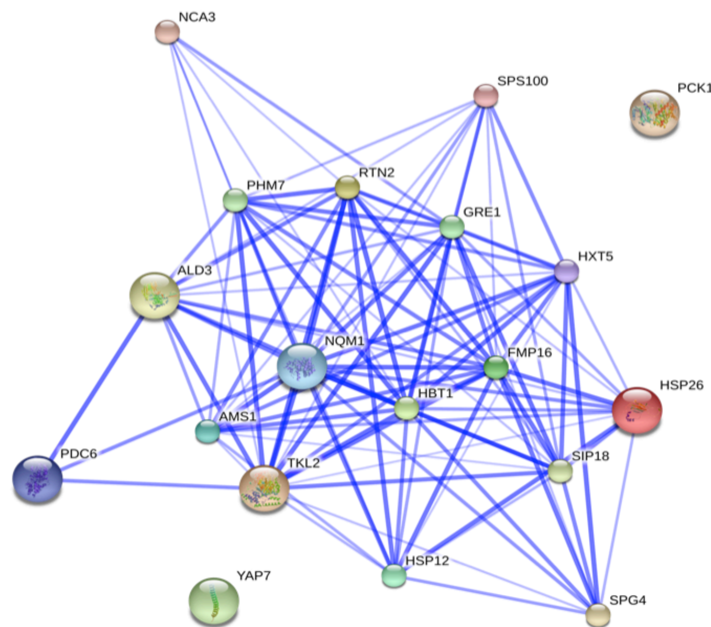

Figure 15. STRING analysis of the "rise and fall" genes. Gene set containing the "rise and fall" genes were subjected to protein-protein interaction prediction using STRING 9.0 (string-db.org). Blue lines indicate interaction between two proteins (nodes). Thicker lines indicate stronger interaction.

In correlating the calcium phenotype of the yeast strains with the expression pattern of genes, two trends in gene expression were considered: a) the "rise and fall" patterned expression exemplified by the aforementioned genes and b) the opposing, "fall and rise" expression pattern characterized by the repression and induction of gene expression in the *ptp2*Δ*msg5*Δ double disruptant and *ptp2*Δ*msg5*Δ*ssk2*Δ triple disruptant, respectively. From our microarray analysis, we were not able to find highly fluctuated genes bearing the "fall and rise" trend in gene expression thus; only the "rise and fall" genes were selected for further analysis.

3.3.3 "Rise and fall" genes share several transcription factors that may be involved in the suppression of the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant by *SSK2* disruption

We inferred that the genes with "rise and fall" patterned expression belong to a common pathway that is responsible for regulating their expression and thus, the cellular process they control. Therefore, we set to find the transcription factors that may be involved in calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant and its suppression by *ssk2*Δ disruption. The list of potential transcription factors related to each "rise and fall" gene was collected from the YEASTRACT (Yeast Search for Transcriptional Regulators and Consensus Tracking) website (http://www.yeastract.com) after which the transcription factors shared by the majority of the genes were determined. Overlaps among some but not all of genes were common for each transcription factor listed (Table 10). Among the "rise and fall" genes, Aft1 was shared by 94.7% (18/19), Rpn4 by 84.2% (16/19), Msn2 by 78.9% (15/19), Sok2 by 78.9% (28/42) and Ste12 by 78.9% (15/19) (cut-off was set at 75%). The presence of various overlaps indicated that some transcription factors might be shared among the different clusters thus; we inferred that Aft1, Rpn4, Msn2, Sok2 and Ste12 might play important roles

in the suppression mechanism of the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant by *ssk2*Δ disruption. However, we do not discount the possibility of other transcription factors being involved in the calcium-induced pathway in our strain.

Table 10. Transcription factors shared by the "rise and fall" genes

Transcription factor	%	ORF/Genes
Aft1	94.7	*HSP26, TKL2, RTN2, HBT1, FMP16, HSP12, AMS1, NQM1, PDC6, HXT5, SPS100, NCA3, PCK1, SPG4, ALD3, SIP18, PHM7, GRE1*
Rpn4	84.2	*HSP26, TKL2, RTN2, HBT1, FMP16, HSP12, AMS1, NQM1, PDC6, HXT5, SPS100, NCA3, PCK1, SPG4, ALD3, PHM7,*
Msn2	78.9	*HSP26, TKL2, RTN2, HBT1, FMP16, HSP12, NQM1, HXT5, SPS100, NCA3, SPG4, ALD3, SIP18, PHM7, GRE1*
Sok2	78.9	*HSP26, HBT1, FMP16, HSP12, AMS1, NQM1, PDC6, HXT5, SPS100, NCA3, PCK1, SPG4, ALD3, SIP18, PHM7*
Ste12	78.9	*HSP26, TKL2, RTN2, HSP12, AMS1, NQM1, PDC6, HXT5, SPS100, NCA3, PCK1, SPG4, ALD3, SIP18, GRE1*

3.3.4 Disruption of *msn2*Δ confers calcium tolerance to the *ptp2*Δ*msg5*Δ double disruptant

To determine which transcription factor is directly involved in the suppression mechanism conferred by *ssk2*Δ disruption, we disrupted the five candidate transcription factors (*AFT1, RPN4, MSN2, STE12, SOK2*) individually in the *ptp2*Δ*msg5*Δ double disruptant background. Results showed that only an additional *msn2*Δ disruption conferred calcium tolerance to the *ptp2*Δ*msg5*Δ double disruptant (Fig. 16). Interestingly, we found that *ptp2*Δ*msg5*Δ*msn4*Δ was calcium sensitive, indicating that Msn2 and Msn4 do not share a redundant function in relation to calcium signaling although Msn4 may play a lesser role. However, the quadruple disruptant *ptp2*Δ*msg5*Δ*msn2*Δ*msn4*Δ showed better growth in the presence of calcium compared to *ptp2*Δ*msg5*Δ*msn2*Δ, suggesting that Msn4 has an additive effect with Msn2 in the regulation of the Ptp2 and Msg5-mediated calcium signaling (Fig.

16).

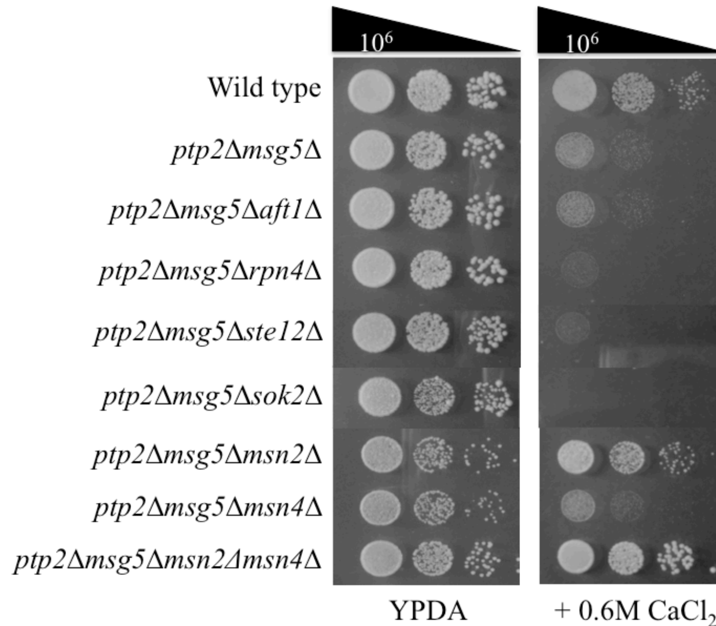

Figure 16. Msn2 mediates the expression of the "rise and fall" genes involved in the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant. Additional *msn2Δ* disruption confers calcium tolerance to the *ptp2Δmsg5Δ* double disruptant. Triple disruptants were constructed by an additional disruption of the candidate transcription factor genes (*AFT1, RPN4, MSN2, STE12, SOK2*) in the *ptp2Δmsg5Δ* background. Only *msn2Δ* disruption was able to confer calcium tolerance to the *ptp2Δmsg5Δ* double disruptant. Yeast strains were grown on YPDA and YPDA + 0.6 M $CaCl_2$ media at 30°C for 2 - 4 days.

To confirm the direct relationship between Msn2 and the suppression mechanism exhibited in the *ptp2Δmsg5Δssk2Δ* double disruptant, we examined the expression of genes with patterned expression in the *ptp2Δmsg5Δmsn2Δ* triple disruptant. We found that the same rise and fall pattern of genes was exhibited in the wild type, *ptp2Δmsg5Δ* and *ptp2Δmsg5Δmsn2Δ* disruptant, indicating a positive correlation between Msn2 and the suppression mechanism conferred by *ssk2Δ* disruption (Fig. 17).

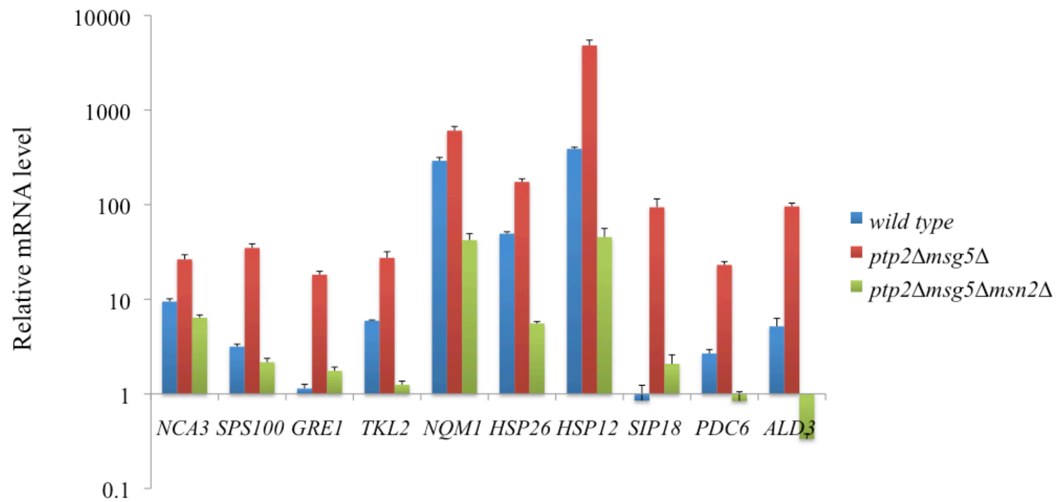

Figure 17. Representative genes showing "rise and fall" patterned expression in the *ptp2Δmsg5Δ* double disruptant and *ptp2Δmsg5Δmsn2Δ* triple disruptant as compared to the wild type. cDNA synthesized from total RNA isolated from the sample strains grown with (0.3 M $CaCl_2$) or without calcium were used as templates for RT-PCR using SYBR Green and specific primers for each gene. *ACT1* was used as an internal control. A "rise and fall" pattern designates the up-regulation of genes in the *ptp2Δmsg5Δ* double disruptant and subsequent down-regulation in the *ptp2Δmsg5Δmsn2Δ* triple disruptant. The results are means of three experiments from three independent preparations.

3.3.5 Constitutive activation of PKA suppresses the calcium sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant

We hypothesized that finding their common upstream regulator can consolidate the heterogeneity of the "rise and fall" genes involved in the Ptp2 and Msg5-mediated calcium signaling. Previously, we have identified two suppressors of the calcium sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant namely PKase disruptions *mck1Δ* and *yak1Δ,* which have functional relationships with the PKA pathway, a major regulator of cell growth (Hermansyah et al., 2010, Santangelo, 2006). Furthermore, published reports describe genetic interactions between PKA components and the candidate transcription factors Aft1, Rpn4, Msn2, Sok2 and Ste12 described above (Ward et al., 1995; Robertson et al., 2000; , Wang et al., 2004; Smith et al., 1998; Pan and Heitman, 1999). Since PKA promotes cell growth, we disrupted the PKA regulatory subunit *BCY1* in the *ptp2Δmsg5Δ* background to determine if

PKA activation can suppress the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant. Interestingly, genetic analysis showed that the *ptp2Δmsg5Δbcy1Δ* triple disruptant exhibited calcium tolerance thus indicating that Bcy1 contributes to the calcium sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant, more likely by inhibiting PKA (Fig. 18).

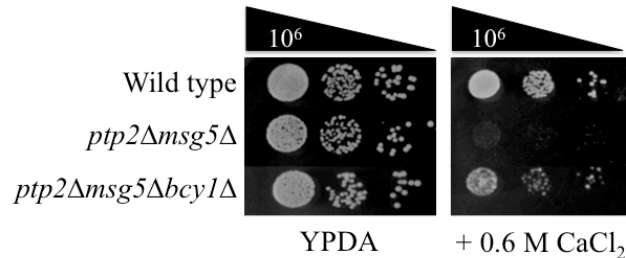

Figure 18. Constitutive PKA activation suppresses the calcium sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant via the regulation of the "rise and fall" genes. Additional *bcy1Δ* disruption in the *ptp2Δmsg5Δ* double disruptant background confers calcium tolerance. *ptp2Δmsg5Δbcy1Δ* disruptant was constructed by disrupting *BCY1* in the *ptp2Δmsg5Δ* double disruptant background by direct gene disruption. Yeast strains were grown on YPDA and YPDA + 0.6 M $CaCl_2$ media at 30°C for 2 - 4 days where *ptp2Δmsg5Δbcy1Δ* disruptant displayed calcium tolerance.

To determine whether the suppression mechanism of *bcy1Δ* disruption is directly related to Ssk2 and the "rise and fall" genes, we checked the patterned expression in the calcium tolerant *ptp2Δmsg5Δbcy1Δ*. Results showed that the expression of the representative genes were also down-regulated in the *ptp2Δmsg5Δbcy1Δ* disruptant thereby showing the same "rise and fall" pattern as in the *ptp2Δmsg5Δssk2Δ* disruptant (Fig. 19). Based on this, we conclude that the suppression mechanism of the *bcy1Δ* disruption is related to Ssk2 through its regulation of the "rise and fall" genes.

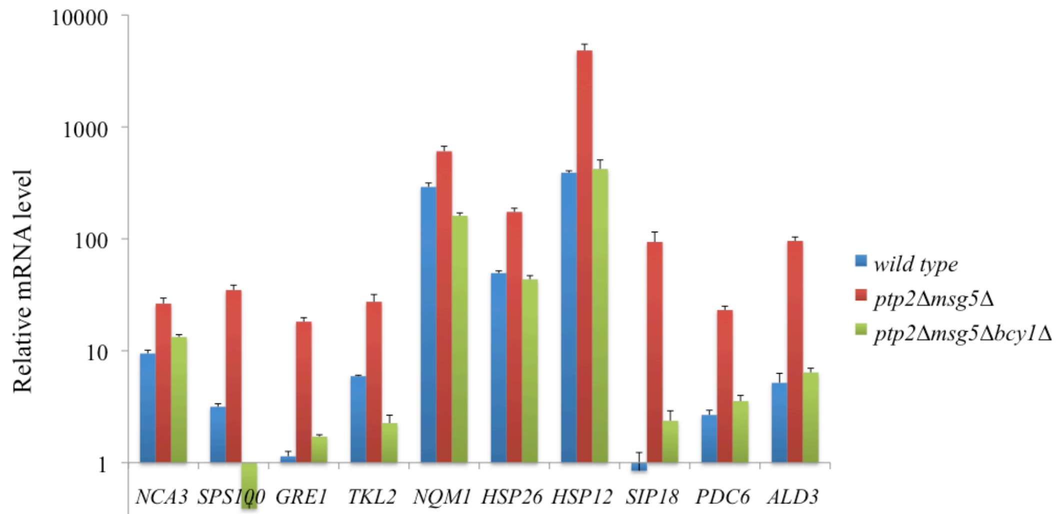

Figure 19. Representative genes showing "rise and fall" patterned expression in the *ptp2Δmsg5Δ* double disruptant and *ptp2Δmsg5Δbcy1Δ* triple disruptant as compared to the wild type. cDNA synthesized from total RNA isolated from the sample strains grown with (0.3 M $CaCl_2$) or without calcium were used as templates for RT-PCR using SYBR Green and specific primers for each gene. *ACT1* was used as an internal control. A "rise and fall" pattern designates the up-regulation of genes in the *ptp2Δmsg5Δ* double disruptant and subsequent down-regulation in the *ptp2Δmsg5Δbcy1Δ* triple disruptant. The results are means of three experiments from three independent preparations.

3.4 Discussion

The non-suppressor nature of the disruptions of other HOG pathway protein kinases implies that the reduced Hog1 phosphorylation in the *ptp2*Δ*msg5*Δ*ssk2*Δ triple disruptant is not a major factor in the suppression of the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ disruptant rather it is a result of an attenuated HOG signaling response due to the disruption of the HOG MAPKKK component *SSK2.* Furthermore, the observed increase in Hog1 phosphorylation in the *ptp2*Δ*msg5*Δ double disruptant was probably due to the absence of the HOG regulator Ptp2 and was not the major cause of the calcium sensitive phenotype.

Our analysis revealed that Aft1, a transcription factor involved in iron utilization and homeostasis as well as oxidative response, may have a role in the suppression mechanism by *ssk2*Δ disruption (Yamaguchi-Iwai et al., 1995; Shakoury-Elizeh et al., 2004; Castells-Roca et al., 2011). It is interesting to note the involvement of iron homeostasis in the calcium-induced signaling pathway of the *ptp2*Δ*msg5*Δ double disruptant since null mutation of *AFT1* is known to cause delayed cell cycle progression in G1 phase similar to the calcium sensitive phenotype of the *ptp2*Δ*msg5*Δ double disruptant (White et al., 2009; Hermansyah et al., 2009). Rpn4 was previously reported to be involved in the expression of proteosomal genes and calcineurin-mediated degradation of Yap1 that occurs to ensure a G2 delay in response to calcium stress. In particular, Yap1 negatively regulates Swe1 and Cln2 indirectly via the down-regulation of *RPN4* expression (Yokohama et al., 2006). Assuming that the up-regulation of the “rise and fall” genes is due to the proteasome activity modulated by Yap1 and Rpn4, Ptp2 and Msg5 may both act as negative regulators of Yap1 while Ssk2 promotes Yap1 and Rpn4 activity. On the other hand, Sok2 is a nuclear protein in the cyclic AMP (cAMP)-dependent protein kinase A (PKA) signaling pathway that negatively regulates pseudohyphal differentiation while Ste12 is a transcription factor related to mating pheromone pathway (Ward et al., 1995; Pan and Heitman, 2000; Santangelo, 2006). We

recently reported the synergistic roles of both Sok2 and Ste12 in the up-regulation of the cell wall genes of the calcium-sensitive *ptp2*Δ*msg5*Δ double disruptant thus; their involvement in the suppression mechanism conferred by *ssk2*Δ disruption is not surprising (Laviña et al., 2013). YEASTRACT analysis revealed that 78.9% (15/19) of the "rise and fall" genes contained one or more STRE binding motif (CCCCT or AGGGG), indicating the commonality of the general stress response transcription factors Msn2 and Msn4. Presumably, Msn2 and Msn4 play a role in mediating the stress response genes (*HSP26, HSP12, ALD3, SIP18* and *GRE1*) that exhibit the "rise and fall" patterned expression in the *ptp2*Δ*msg5*Δ double disruptant. In this case, Msn2 and Msn4 may promote induction of the stress response genes that eventually leads to a calcium sensitive phenotype.

It has been widely accepted that Msn2 is a stronger transcription factor than Msn4 although both share functional similarities (Hohmann, 2002). Our data support this idea wherein disruption of *MSN2* alone can confer calcium tolerance while *msn4Δ* cannot. However, Msn2 and Msn4 were found to exhibit an additive effect as exemplified by the better growth of the *ptp2*Δ*msg5*Δ*msn2*Δ*msn4*Δ quadruple disruptant in YPDA supplemented with calcium (Fig. 16).

One possibility is that the calcium sensitivity was a result of growth inhibition initiated by Yak1 since *yak1*Δ disruption had the most profound change on the phenotype from calcium sensitive to tolerant (Hermansyah et al., 2010). In this case, we assumed that constitutive PKA activation (due to *bcy1*Δ disruption) was able to suppress the cell cycle arrest by overcoming Yak1 activity. Thus, we hypothesized that PKA served as an upstream regulator of the calcium-induced signaling pathway exhibited in the *ptp2*Δ*msg5*Δ double disruptant. We have also observed a similar "rise and fall" pattern in the *ptp2*Δ*msg5*Δ*yak1*Δ triple disruptant, prompting us to conclude that the suppression mechanism conferred by *ssk2*Δ has a direct relationship with PKA componentsYak1 and Bcy1 (Fig. 20). In addition,

PKA is known to phosphorylate at least five sites on Msn2 to prevent its activity (Gorner et al., 2002). This may indicate that the suppression mechanism of the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant through constitutive PKA activation (*bcy1*Δ disruption) may also occur via the inhibition of Msn2 (and Msn4) activity.

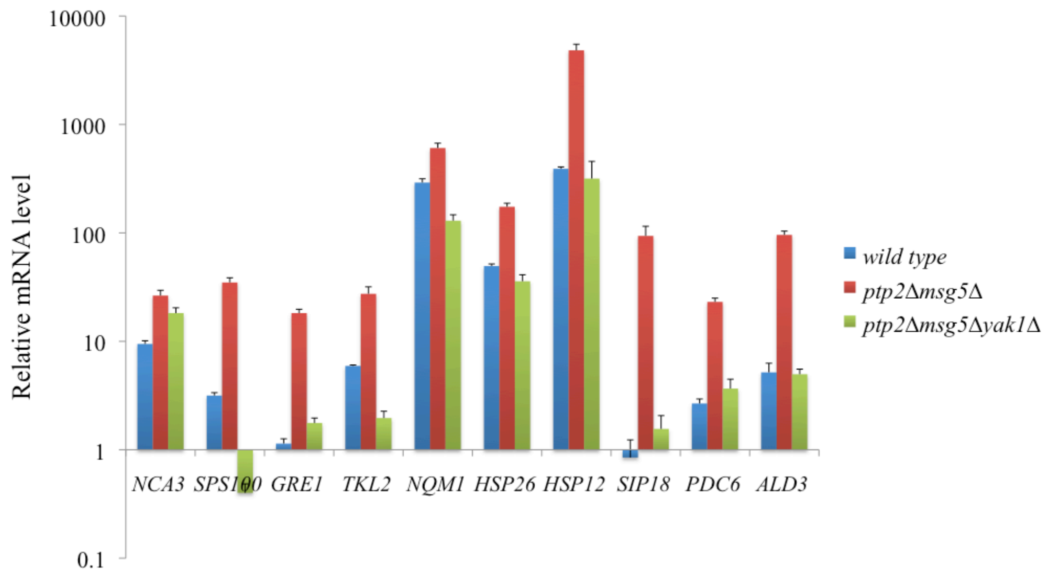

Figure 20. Representative genes showing "rise and fall" patterned expression in the *ptp2*Δ*msg5*Δ double disruptant and *ptp2*Δ*msg5*Δ*yak1*Δ triple disruptant as compared to the wild type. cDNA synthesized from total RNA isolated from the sample strains grown with (0.3 M $CaCl_2$) or without calcium were used as templates for RT-PCR using SYBR Green and specific primers for each gene. *ACT1* was used as an internal control. A "rise and fall" pattern designates the up-regulation of genes in the *ptp2*Δ*msg5*Δ double disruptant and subsequent down-regulation in the *ptp2*Δ*msg5*Δ*yak1*Δ triple disruptant. The results are means of three experiments from three independent preparations.

In conclusion, the calcium sensitivity and the subsequent tolerance conferred by the protein kinase suppressors show the complexity of signaling cascades present in eukaryotic cells. Specifically, the yeast cells possess a number of redundant proteins and pathways to combat inadvertent activation or repression of pathway signals. As we have shown previously, such is the case for our *ptp2*Δ*msg5*Δ double disruptant strain in response to high intracellular calcium where we found the redundant function of the SLT2 and calcineurin pathways (Laviña et al., 2013). Furthermore, the absence of the functionally redundant *PTP2*

and *MSG5* has proven to be detrimental to cells exposed to high calcium concentrations. Based on our results, *PTP2* and *MSG5* are integral in maintaining signal specificity thus their absence can lead to cross-talk events involving several pathways. In this study, we found another alternative pathway that mediates the calcium phenotype of the *ptp2*Δ*msg5*Δ double disruptant involving a novel function of Ssk2 that is related to PKA-related proteins Yak1 and Bcy1, transcription factor Msn2 as well as a group of calcium-induced, stress response-related genes.

Chapter 4

Conclusion and general discussion

S. cerevisiae possesses an inherent ability to cope with the deleterious changes in the environment by activating a variety of signaling cascades that control patterns of gene expression and protein activity. Since inappropriate activation of cell signaling pathways often has dire consequences on the viability, cells possess a myriad of ways to combat improper activation of signaling pathways, one of which is regulation via reversible phosphorylation mediated by protein kinases and phosphatases. In an effort to study the functional redundancy of protein phosphatases in yeast, Sakumoto and colleagues (2002) constructed a PPase library of single and double disruptants and systematically screened for interesting phenotypes; one of which is that the disruption of two protein phosphatase genes, *PTP2* and *MSG5*, causes calcium sensitivity indicating that functional redundancy exists between the two PPases in response to high extracellular calcium. Furthermore, we found that additional disruption of protein kinases *BCK1, MKK1, SLT2, MCK1, YAK*1 and *SSK2* in the *ptp2*Δ*msg5*Δ background confers calcium tolerance.

This work describes the suppression mechanism conferred by the protein kinase disruptions. First, we discuss the suppression mechanism of the SLT2 pathway suppressors *bck1*Δ, *mkk1*Δ and *slt2*Δ as having functional redundancy with the calcineurin pathway. In the event of a calcium-induced, calcineurin-driven signaling pathway activation, the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant can be suppressed by preventing over-activation of the calcium-induced signaling cascade under the parallel control of the SLT2 and calcineurin pathways via inactivating either one of the two (Fig. 10).

In our study we have discovered functional redundancy existing at two levels: at the protein level between Ptp2 and Msg5 and at the cascade level between SLT2 and calcineurin pathways. At the protein level, Ptp2 and Msg5 have redundant functions as negative

regulators of the SLT2 pathway. This indicates the importance of preventing the hyper-activation of the calcium-induced signaling pathway mediated by the parallel SLT2 and calcineurin pathways. In our model, calcineurin is the primary signaling cascade activated upon calcium exposure while SLT2 acts as an alternate, secondary pathway if calcineurin is impaired. Thus, in the normal calcium-exposed setting, SLT2 pathway should be repressed via negative regulation by Ptp2 or Msg5. At the cascade level, we show the functional redundancy between SLT2 and calcineurin pathways in response to high extracellular calcium (Fig. 10). Conversely, functional redundancy also serves as safeguard against the lethal effects of an inactive signaling pathway. It is believed that genetic redundancy contributes to the genetic robustness of yeast cells against external changes as shown in fitness data with significantly higher probability of functional compensation for a duplicate gene (Gu et al., 2003) However, this theory contradicts population genetics prediction that genetic redundancy is evolutionary unstable (Li et al., 2010). Our data more likely supports the theory of genetic buffering which results in the concealment of phenotypic consequences of mutations. In fact, redundancy is the best characterized form of buffering relationship, where genes can compensate for the loss of another by their ability to share and takeover the same function (van Wageningen et al., 2010).

In our model, we hypothesized that the calcium-induced signaling pathway mediated by the parallel SLT2 and calcineurin pathways bottlenecks at Crz1 since *crz1Δ* in the *ptp2*Δ*msg5*Δ background resulted in a calcium sensitive phenotype (Fig. 10). It has been reported that Crz1 is the major, if not the only, effector of the calcineurin-regulated gene expression in yeast (Yoshimoto et al., 2002). However, we did not find correlation between normally activated Crz1 targets and the Ptp2 and Msg5-mediated calcium signaling thus, we deduced that in this particular signaling cascade, Crz1 targets a different group of downstream genes. Currently, the identity of the Crz1 targets is still undetermined.

We have also predicted another alternative pathway that can suppress the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant that involves the PKase suppressors *ssk2*Δ and *yak1*Δ based on FACS analysis. Genome-wide transcriptional analysis of the *ptp2*Δ*msg5*Δ and *ptp2*Δ*msg5*Δ*ssk2*Δ disruptants revealed 19 genes with a "rise and fall" patterned expression that is related to the calcium phenotype. We discovered that these genes were mediated by the transcription factor Msn2 (and partly by Msn4) which served as the convergence point between Ssk2 and PKA since this particular group of suppressors (*ssk2*Δ, *yak1*Δ, *bcy1*Δ and *msn2*Δ) all exhibit the "rise and fall" patterned expression of effector genes.

In detail, upon exposure to high extracellular calcium, Ssk2 is responsible for the induction of 19 "rise and fall" genes that contribute to the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant via Yak1 and the transcription factor Msn2. Suppression of the induction of the "rise and fall" genes can occur by inactivating any component of the linear cascade namely Ssk2, Yak1 or Msn2. In addition, inhibiting Msn2 activity by constitutive PKA activation (*bcy1*Δ disruption) also results in the repression of the "rise and fall" genes. However, the positions of Ssk2 and Yak1 have been arbitrarily placed in the linear pathway and the exact position of the proteins in the Ptp2 and Msg5-mediated calcium signaling is the subject of future experiments (Fig. 21).

Using genetic and transcriptional analyses, the roles of the previously isolated PKase suppressors *bck1*Δ, *mkk1*Δ, *slt2*Δ, *ssk2*Δ and *yak1*Δ in the calcium-induced, Ptp2 and Msg5-mediated signaling in *S. cerevisiae* were clarified. In addition to the previously identified PKase suppressors, additional suppressors namely *cnb1*Δ, *bcy1*Δ and *msn2*Δ were identified in this study. It also provides evidence to the existence of several alternative pathways that mediate calcium signaling in the *ptp2*Δ*msg5*Δ double disruptant.

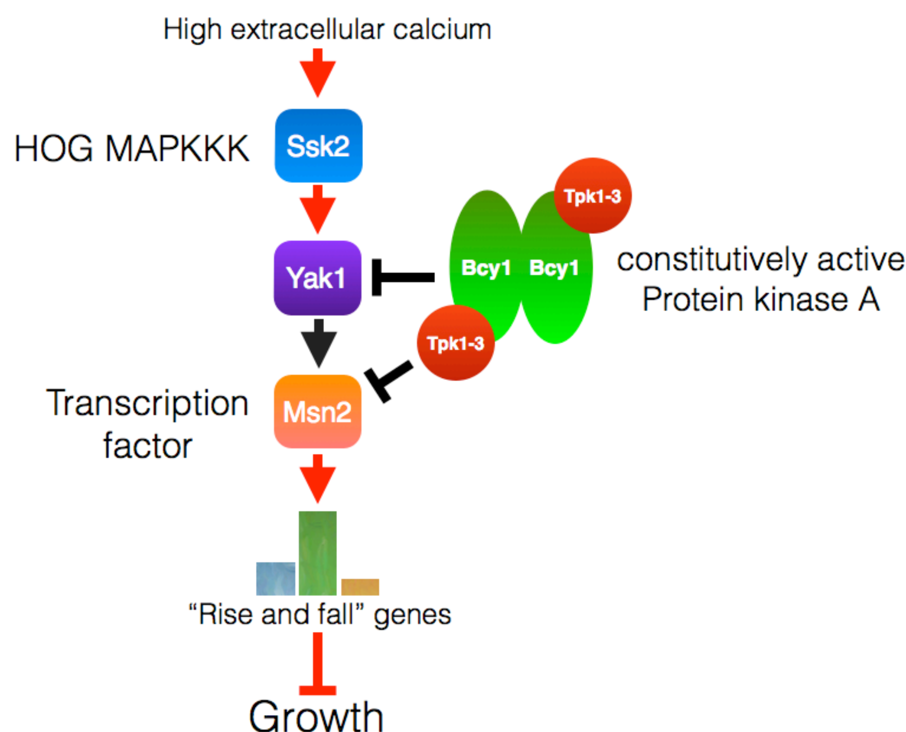

Figure 21. Suppression of the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant results from the repression of the Msn2-mediated "rise and fall" genes. High extracellular calcium triggers the induction of 19 "rise and fall" genes responsible for the calcium sensitivity of the *ptp2*Δ*msg5*Δ double disruptant via Ssk2, Yak1 and the transcription factor Msn2. Repression of the "rise and fall" genes can be achieved by disruption of any component of the linear pathway (*ssk2*Δ*, yak1*Δ*, msn2*Δ) or constitutive PKA activation (*bcy1*Δ).

In our model, Msn2 was placed downstream of Ssk2 based on their roles in the HOG pathway. However, a direct genetic relationship between Ssk2 and Msn2 was not established in our study. Furthermore, there are no published reports of a direct interaction between Ssk2 and Msn2 (BioGRID), indicating the possibility of an unknown Ssk2 substrate that mediates Msn2 regulation.

This study shows evidence of multi-level, functionally redundant mechanisms in *S. cerevisiae* responsible for buffering the lethal effects of genetic and environmental changes. Here, we have outlined a case where the detrimental effects of gene deletion were circumvented by the activation or deactivation of alternative pathways involving SLT2, calcineurin, PKA and a novel function of Ssk2.

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