UNIVERSITY OF GOTHENBURG

Integrative analysis of osmoregulation in yeast *Saccharomyces cerevisiae*

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Abstract

Similar to other unicellular organisms, yeasts frequently encounter environmental stress such as heat shock, osmotic stress, and nutrition limitations, which challenge their growth potential. To survive, all living cells must be able to adapt to changes in their surrounding environment. A set of adaptive responses is triggered that leads to repair of cellular damage in order to overcome these stress conditions. The aim of this thesis is to determine how yeast cells respond to changes in osmolarity and water activity.

Upon hyperosmotic shock, water flows out of the cell, resulting in cell shrinkage, and consequently an increase in the concentrations of all substances present in the cytoplasm. Cells adapt their internal osmolarity by gaining an appropriate cell volume as well as an internal water concentration that is optimal for biochemical processes to recover turgor pressure. Osmoregulation is an active process which is mainly regulated by the High Osmolarity Glycerol (HOG) pathway and controls the cellular water balance.

The HOG pathway is one of the four yeast MAP kinase pathways. It conveys the hyper osmolarity stress stimulus into the cell machinery and instigates appropriate responses, including global readjustment of gene expression, changes in translational capacity, transient cell cycle arrest, and accumulation of the compatible solute glycerol. Together, these processes result in osmoadaptation.

In this thesis I investigated the quantitative characteristics of osmoregulation in the yeast *Saccharomyces cerevisiae*. I applied a combination of traditional molecular approaches and frontline technologies for comprehensive and quantitative measurements, such as high throughput experiments, synthetic biology, single cell analysis and mathematical modeling to understand the interdependence and timeline of different osmoadaptation process.

List of Papers

This thesis based on the following papers:

I. Babazadeh R, Adiels CB, Smedh M, Petelenz-Kurdziel E, Goksör M, Hohmann S (2013) Osmostress-Induced Cell Volume Loss Delays Yeast Hog1 Signaling by Limiting Diffusion Processes and by Hog1-Specific Effects. PLoS ONE 8(11): e80901. doi:10.1371/journal.pone.

II. Babazadeh R, Furukawa T, Hohmann S & Furukawa K. (2014) Rewiring yeast osmostress signaling through the MAPK network reveals essential and non-essential roles of Hog1 in osmoadaptation. Scientific Reports, Sci Rep. 2014 Apr 15;4:4697. doi: 10.1038/srep04697.

III. Rastgou Talemi S*, Tiger C.F*, Babazadeh R, Andersson M, Klipp E, Hohmann S, Schaber J. Systems Biology Analysis of the Yeast Osmo-Stat. Manuscript.
* Equal contribution

IV. Ahmadpour D, Babazadeh R, Andersson M, Maciaszczyk-Dziubinska E, , Dahal S, Wysocki R, Tamás M.J, Hohmann S. The MAP kinase Slt2 modulates transport through the aquaglyceroporin Fps1. Manuscript.

V. Babazadeh R, Lahtvee P-J, Adiels CB, Goksör M, Nielsen J.B, Hohmann S. The yeast osmostress response is carbon source dependent. Manuscript.

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1 Introduction

Similar to other organisms, especially unicellular organisms, yeast cells are constantly exposed to various forms of stress such as heat shock, osmotic stress, and nutrition limitations. Cells must respond to environmental changes in order to maintain their viability and proliferation rate. Robustness is an intrinsic feature of biological systems, which allows them to be adaptable to external changes. The adaptive responses to different stress factors are mediated by specific signal transduction pathways.

Changes in osmolarity and water activity can occur both slowly and rapidly in yeast's natural environment. The HOG pathway is one of the three yeast MAP kinase pathways. It conveys the hyper osmolarity stress stimulus into the cell machinery and instigates appropriate responses, including global readjustment of gene expression, changes in translational capacity, transient cell cycle arrest, and accumulation of the compatible solute glycerol. Together, these processes result in osmoadaptation.

To achieve an integrated understanding of osmoadaptation, it is important to elucidate the interdependence of different events that occur during osmoadaptation. In addition to information about components and pathways involved in osmoadaptation, we also need to address issues regarding feedback control of HOG pathway activity, timeline of events, cross-regulation of HOG pathway by other MAPK pathways, and signal fidelity in order to reach an inclusive view of the dynamics of the underlying adaptation process.

My research concerns mechanisms that control the yeast HOG signaling pathway in response to hyperosmotic stress. This thesis studies quantitative characteristics of HOG pathway regulation and how the Cell Wall Integrity (CWI) pathway cooperates in osmoadaptation, as well as the roles of different metabolic pathways in osmoregulation of ethanol-grown cells.

2 Molecular biology versus systems biology

Molecular biology studies the function of single molecules individually. However, a biological system is not just an assembly of genes and proteins and consequently the properties of a system cannot be fully understood by illustrations of its interconnections (Kitano 2002b). Instead, the structure and dynamics of cellular function need to be examined in order to elucidate how organisms function as a whole. In fact, both specific elements and a combination of the network are involved to define functions in biological systems (Kitano 2002a). Yet, the main challenge for biology is to understand and explain the principles and mechanisms of system's behavior (Kitano 2002b; Stelling 2004; Bruggeman and Westerhoff 2007). Developments in molecular biology, especially genome sequencing and high-throughput measurements such as genomics and proteomics allow us to collect inclusive data sets on system performance and shift the focus of research from molecules to networks (Stelling 2004; Bruggeman and Westerhoff 2007).

Systems biology integrates experimental biology with mathematical modeling and employs rules of chemistry and physics to explain the properties of biological systems. Generation of quantitative and time-resolved data required for mathematical modeling entails collaboration between biology, physics, and chemistry (Ehrenberg et al. 2009). Two major methodological approaches have been established in systems biology in order to explain the behavior of biological networks: (1) data-driven or top-down systems biology and (2) module-driven, aka data-requiring, or bottom-up systems biology.

In top-down systems biology, a new model for a molecular mechanism is constructed based on experimental data, such as protein interaction networks, genetic interaction networks, metabolite maps, phosphorylation networks, and gene expression networks (Ehrenberg et al. 2009). The data are then analyzed and integrated with mathematical modeling in order to define correlations between concentrations of molecules. It concludes with formulation of hypotheses regarding regulation of clusters of the studied molecules. These hypotheses predict new correlations, which can be examined with a new set of experiments (Bruggeman and Westerhoff 2007).

In contrast, bottom-up systems biology is based on formulating the interactive behavior of a manageable part of the system, such as rate equation of an enzymatic process (Bruggeman and Westerhoff 2007). Although these mathematical models only contain a

limited number of components in a defined cellular module, they provide tools for analysis of processes over time. In this approach the main objective is to integrate different pathway models into a model for the entire system level (Bruggeman and Westerhoff 2007; Ehrenberg et al. 2009). However, it is crucial that a suggested phenomenon predicted by this model is actually confirmed by experimental methods (Bruggeman and Westerhoff 2007).

Though, it seems neither of these two approaches can explain heterogeneity or noise, which is a feature of all living cells and can have strong effect on the behavior at system level (Walker and Southgate 2009). In fact, even in a population of genetically identical cells, there are variations among individual cells due to the stochastic or random nature of chemical reactions. Such heterogeneity can be beneficial for many biological processes, while fidelity in cellular behavior is required (Maheshri and O'Shea 2007). This variability arises either from gene expression or fluctuations in cellular components, which produces intrinsic or extrinsic noises respectively (Elowitz et al. 2002; Raser and O'Shea 2004). Therefore, a third approach was developed, called “middle-out”, which relies on individual-based models and currently available information (Walker and Southgate 2009). The improvement of tools for single cell analysis that allow detecting variations between individual cells and monitoring processes in real time provides a comprehensive amount of data for the middle-out approach.

Robustness is an inherent feature of biological systems. It is defined as the ability of a system to preserve phenotypic stability when facing various perturbations, including internal and external changes (Kitano 2002a; Stelling et al. 2004). Robustness is a dynamic process, which makes a system relatively insensitive to internal alterations and adaptable to changes in environment as well (Stelling et al. 2004). This robustness is attained by feedback, modularity, redundancy, and structural stability tactics (Kitano 2002b).

Feedback control plays a critical role in preservation of cellular functions. In fact, feedback control monitors a system and regulates the output of a reaction and similarly controls appropriate input signals (Freeman 2000; Stelling et al. 2004). In negative feedback, the final, or any intermediate, product of a reaction regulates upstream component by inhibition. Therefore, negative feedback reduces the difference between actual output and the set point and increases the stability of the system. However, in

positive feedback, increasing the output leads to boosting the upstream response (Becskei and Serrano 2000; Stelling et al. 2004). Another feature of robust systems is modularity, where any subsystem has separate functions from other subsystems. This separation is achieved through chemical isolation, which is derived from spatial localization or chemical specificity, and prevents spread of a failure in one module to other parts (Hartwell et al. 1999; Kitano 2002b). Redundancy is known as the simplest strategy to increase the robustness of a system and occurs while several independent units perform the same function (Hartman et al. 2001; Kitano 2002a).

Theoretical and experimental analysis have both confirmed that various processes that occur in a biological system, such as signaling pathway regulation (Huang and Ferrell 1996; Lee et al. 2003) and cell cycle regulation (Borisuk and Tyson 1998; Morohashi et al. 2002; Pomerening et al. 2003), display characteristics of a robust behavior. Therefore, the principle of robustness is an essential concept in systems biology, which is applied in studying dynamic networks when their kinetic parameters are mainly unidentified. Common parameter properties in robust systems have allowed for creating a model that comprises only few known regulatory proteins (Von Dassow and Odell 2002; Stelling et al. 2004).

Taken together, combination of mathematical modeling and engineering with new technologies for comprehensive and quantitative measurements, such as high throughput experiments, synthetic biology, and single cell analysis, have linked our knowledge at molecular level with system-level understanding. This approach aims to understand biological systems by identifying their structures and dynamics, in order to control cellular behaviors under external stimuli.

3 Single cell analysis versus population analysis

Traditional molecular biology is based on experimental approaches, which treats individual cells with uniform environments. They are, however, analyzed and presented as a blended average parameter (Di Carlo and Lee 2006). Bulk methods such as Western, Northern, and Southern blotting, as well as microarrays, which are applied to determine gene expression profile or protein levels are well-established techniques. However, they are not able to reflect the correct distribution of a response required to understand cellular behavior (Teruel and Meyer 2002; Lidstrom and Meldrum 2003). This means that the kinetics of a response and the average value of data obtained from population studies can be misinterpreted due to heterogeneity within the population (Di Carlo and Lee 2006). Single cell studies based on reporter gene technology have demonstrated cellular heterogeneity in both prokaryotic (Ozbudak et al. 2002; Mettetal et al. 2006) and eukaryotic cells (Blake et al. 2003). As mentioned, this heterogeneity originates from gene expression and also from fluctuations in different cellular components (Elowitz et al. 2002; Raser and O'Shea 2004). This can be explained by the fact that, at any given time, cells are in different stages of the cell cycle, which could impact their physiological state and influence their features accordingly (Spudich and Koshland 1976; Sott et al. 2008). Therefore, it is important to know how the behavior of single cells influences the internal cellular processes. Also how similar single cells are in their biochemical characteristics (Spudich and Koshland 1976). Single cell analysis appears to be an informative approach to answer biological questions. However, single cell data must be analyzed based on the preliminary population data to prevent any misinterpretation. Furthermore, it is important to perform single cell experiments on a statistically significant number of cells since interpreting the behavior of just a few individual cells may lead to false conclusions (Sott et al. 2008).

4 Yeast HOG pathway as a model system

Yeast cells are unicellular fungi which live on plant or animal material; hence they are repeatedly exposed to extremely variable stress factors, such as nutrients starvation, as well as temperature, pH and especially water activity changes. Yeast cells have developed mechanisms to adapt to such shifting environmental conditions through different mitogen-activated protein kinase (MAPK) pathways in order to maintain viability and proliferation capability (Marshall 1994; Gustin et al. 1998).

The yeast HOG (High Osmolarity Glycerol) pathway is a well-studied MAPK cascade that mediates cellular response to hyperosmotic stress (Gustin et al. 1998). The Principles of osmoadaptation are conserved across eukaryotes (Brewster et al. 1993; Gustin et al. 1998). Also osmotic changes can be well controlled; therefore the yeast HOG pathway has become a powerful eukaryotic model. To date, experimental observations and mathematical modeling have elucidated specific properties of osmoregulation regarding the role of basal signaling, robustness against perturbation, in addition to adaptation and feedback control (Hohmann 2009).

In this thesis, I applied quantitative biological approaches including population and single cell analysis integrated with mathematical modeling to characterize at a quantitative level HOG pathway response features as well as the control of crosstalk between the HOG and other yeast MAPK pathways.

5 Water activity, volume recovery, and osmoregulation

Similar to other unicellular organisms, the yeast *Saccharomyces cerevisiae* is exposed to an ever-changing, highly variable environment, which may challenge its growth potential. To survive, yeast cells must cope with alterations in nutrients, temperature, pH, and especially water activity (Hohmann 2002b). A set of cellular responses is triggered that leads to an instant repair of cellular damage in order to overcome these stress conditions (Siderius et al. 1997).

Water activity is defined as the chemical potential of free water in solution, and expresses the tendency of water to contribute in biochemical reactions (Hohmann 2002b). Upon hyperosmotic shock, water flows out of the cell, resulting in cell shrinkage, and consequently an increase in the concentrations of all substances present in the cytoplasm. In contrast, upon hypo osmotic stress, water flows into the cell, causing an increase in cell volume, and a decrease in the concentration of solutes. Cells must adapt their internal osmolarity by gaining an appropriate cell volume as well as an internal water concentration optimal for biochemical processes to recover turgor pressure (Blomberg and Adler 1992; Hohmann 2002a). Since water loss or uptake occurs very fast, it is crucial that survival mechanisms function immediately after a sudden osmotic shift (Blomberg and Adler 1992). However, adaptation after a hyperosmotic shock may take several hours (Blomberg and Adler 1992; Hohmann 2002b).

S. cerevisiae has evolved to re-establish its water balance by accumulating the compatible osmolyte glycerol (Hohmann 2002b). A diverse range of molecules can act as compatible osmolytes such as amino acids, polyols and sugars, methylamines, methylsulfonium compounds, and urea (Yancey 2005). Osmolytes function to decrease the intracellular water potential and thereby drawing water into the cell. In addition, osmolytes can have unique protective metabolic roles like acting as antioxidants, providing redox balance, and detoxifying sulfide (Yancey 2005). When cells have accumulated sufficient levels of compatible osmolytes and recovered turgor pressure and also gained an appropriate cell volume, growth can resume in the high osmolarity condition (Figure 1). Adapted cells are more protected when exposed to a new stress situation (Siderius et al. 1997).

Upon hypo osmotic shock (Figure 1), external osmolarity decreases and water enters the cell. Therefore it is important for yeast cells to reduce their intracellular glycerol level and maintain proper turgor pressure (Tamas et al. 1999; Levin 2005).

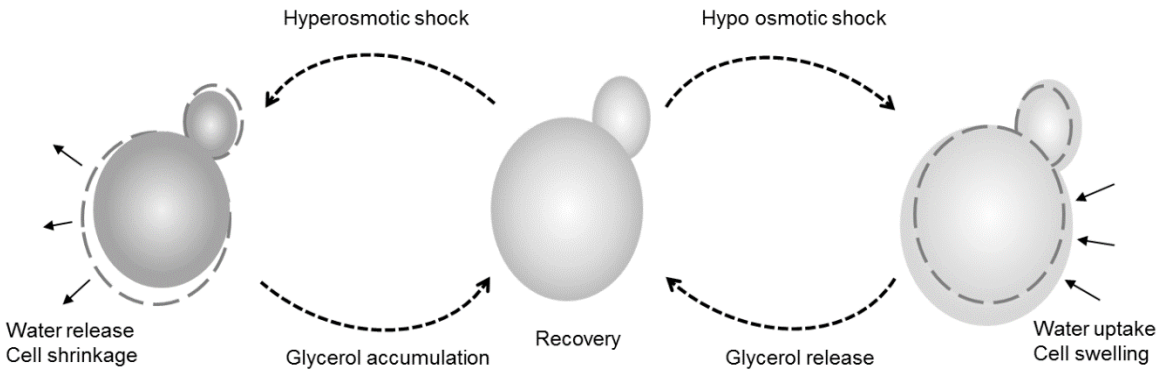

Figure 1. Representation of osmoregulation in yeast *Saccharomyces cerevisiae.*

Upon hyperosmotic stress, water flows out of the cell resulting in an almost immediate decrease in cell volume and consequently an increase in concentration of all substances present in the cytoplasm. Adaptation involves glycerol accumulation and thus creation of an appropriate turgor pressure and cell volume recovery. In contrast, upon hypo-osmotic stress, water flows into the cell causing an increase in cell volume. Adaptation occurs through releasing excessive glycerol in order to prevent bursting and thus balancing the turgor pressure.

The yeast, *S. cerevisiae* responds to external stimuli via mitogen-activated protein kinase (MAPK) pathways. The high osmolarity glycerol (HOG) pathway and the cell wall integrity (CWI) pathway coordinate adaptive responses to high and low osmolarity, respectively (Hohmann 2002b; Levin 2005).

6 Yeast MAPK pathways

MAPK cascades are evolutionarily conserved eukaryotic signaling modules (Chen et al. 2001). MAPK cascades are one type of highly complex pathways that yeast cells utilize in order to respond and adapt to a changing environment (Gustin et al. 1998). The Mitogen Activated Protein Kinase (MAPK) pathways convey a diverse range of signals from the cell surface to initiate proper cytoplasmic and nuclear responses to regulate cell cycle progression, cell growth and morphogenesis, and stress responses (Marshall 1994; Gustin et al. 1998).

The core of MAPK pathways consists of three kinases that act in series including a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAP kinase (MAPK) (Figure 2). The MAPKKK is activated either by phosphorylation through an upstream kinase or by binding of an activator protein. Then, the MAPKKK activates the MAPKK by dual phosphorylation on a serine and a threonine residue. Subsequently, the activated MAPKK phosphorylates the MAP kinase (MAPK) by phosphorylation on a threonine/serine and a tyrosine residue separated by one arbitrary amino acid (Marshall 1994).

Some kinases are involved in more than one MAPK pathway. For instance, the MAPKKK Ste11 participates in the mating, pseudohyphal development, and osmoregulation pathways (Widmann et al. 1999). Although the shared components provide a capacity for signal integration, they may lead to a loss of specificity of a particular response. Therefore, it is crucial for cells to overcome this problem in order to survive. However, these pathways achieve specificity by filtering out spurious crosstalk through mutual inhibition, as well as diverse upstream activation mechanisms that include mechano-sensitive sensors, G-protein-coupled receptors, and phosphorelay systems (Widmann et al. 1999; McClean et al. 2007).

Moreover, the duration and magnitude of the pathways' activation is essential for determining the efficiency of the response (Martin et al. 2005). In fact, inappropriate activation of pathways may cause lethal effects (Maeda et al. 1993) . Therefore, molecular mechanisms that ensure an accurate intensity of signaling and a precise timing of activation are needed. Since phosphorylation of both the threonine and the tyrosine is required for MAPK activity, dephosphorylation of either is sufficient and an effective

mechanism for inactivation. There are three types of protein phosphatase involved in down regulation of MAPK pathways, including protein tyrosine phosphatases (PTPs), protein serine/threonine phosphatases gene (PTCs), and dual-specificity protein phosphatases (DSPs) (Martin et al. 2005).

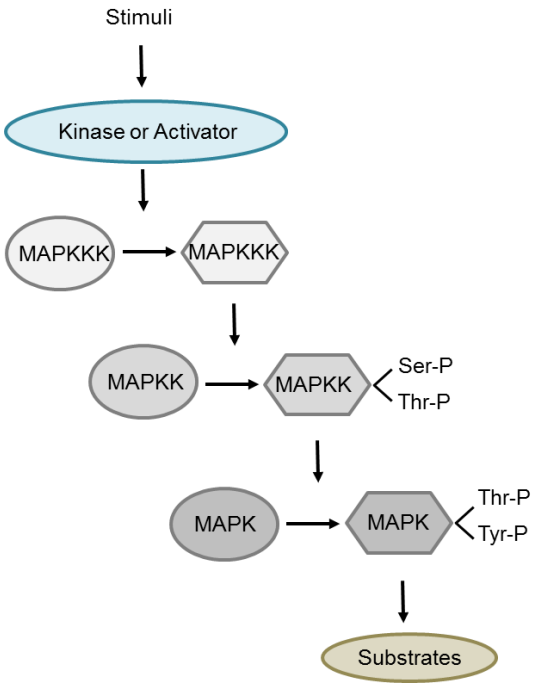

Figure 2. Schematic diagram of the MAP kinase module.

Ellipses and hexagons represent inactive and active forms of kinases, respectively. MAPK, MAP kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase.

There are four MAPK pathways (Figure 3) in yeast *S. cerevisiae*; pheromone pathway, pseudohyphal growth pathway, high osmolarity glycerol pathway, and cell wall integrity pathway (Gustin et al. 1998). In addition, there is another MAPK in yeast, Smk1, which is required in sporulation. Smk1 is phosphorylated by a MAPK-like activation loop. However, Smk1 activation does not seem to require members of the MAPKK family and hence is not regarded as a prototypical MAPK (Gustin et al. 1998; Whinston et al. 2013).

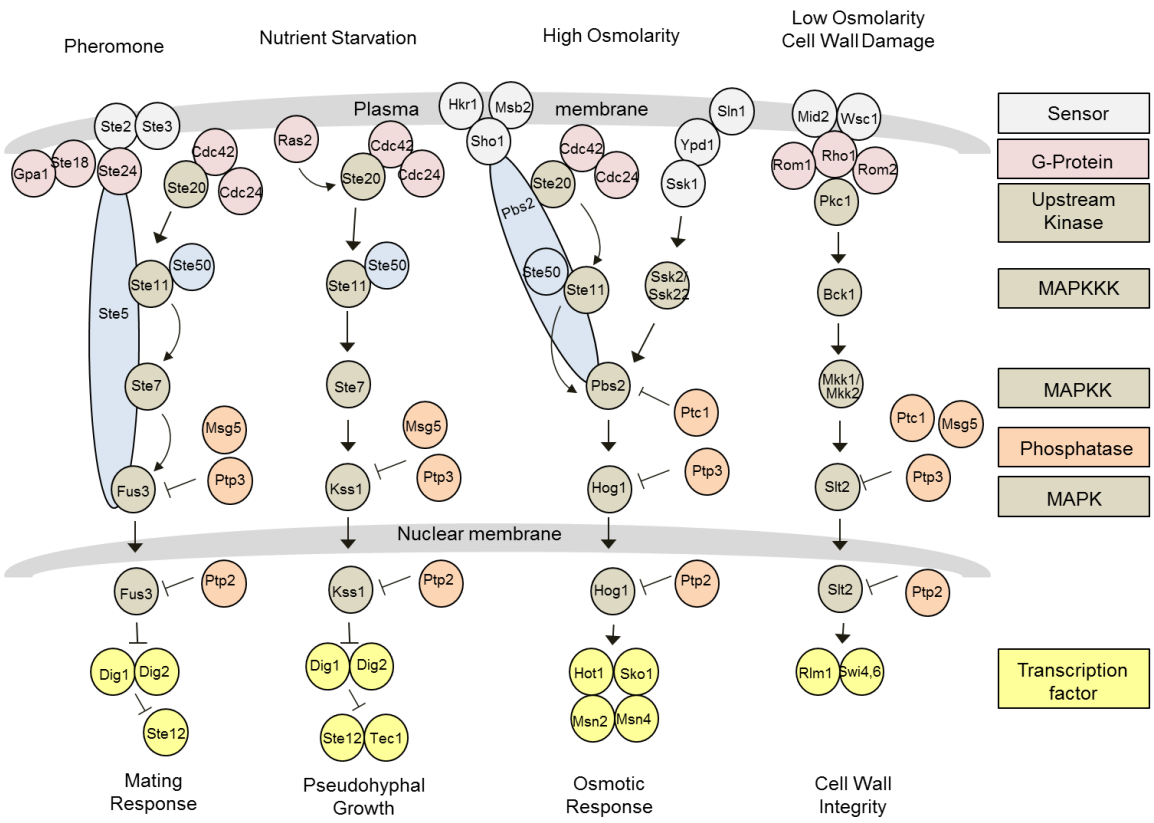

Figure 3. The yeast MAPK pathways.

There are four MAPK pathways in yeast *S. cerevisiae* containing the pheromone pathway, the pseudohyphal growth pathway, the high osmolarity glycerol pathway, and the cell wall integrity pathway. Adapted with courtesy of Professor Stefan Hohmann, University of Gothenburg

The pheromone pathway (Ste11 → Ste7 → Fus3) mediates cellular responses to mating pheromones by Fus3 MAPK. Activated Fus3 regulates the expression of numerous mating-specific- genes by activating the transcription factor Ste12. Fus3 also temporarily arrests cell cycle in G_1, and mediates remodeling of the cytoskeleton and the cell wall and eventually causes cell fusion with the mating partner (Chen and Thorner 2007; Saito 2010). Pheromone stimulation leads to activation of Kss1 through Ste11 and Ste7 (Ma et al. 1995). However, in contrast to Fus3, activation of Kss1 does not require Ste5 scaffolding (Flatauer et al. 2005). Lack of both Fus3 and Kss1 causes sterility, whereas the presence of either is sufficient for mating. This indicates that these MAPKs have a redundant function. However, Fus3 plays the major role in pheromone response (Chen and Thorner 2007).

The pseudohyphal growth pathway (Ste11→ Ste7 → Kss1) mediates adjustments to nutrient limiting conditions by the Kss1 MAPK. When this pathway is activated yeast cells undergo a developmental change called filamentous growth, during which the cells become elongated and mother and daughter cells remain attached to each other, forming filaments of cells called pseudohyphae. The Kss1 MAPK controls cell adhesion, cell elongation, and reorganization of cell polarity through activation of the transcription factors Ste12 and Tec1 (Chen and Thorner 2007; Saito 2010).

The high osmolarity glycerol pathway and the cell wall integrity pathway are discussed in the following sections.

6.1 High osmolarity glycerol pathway (HOG)

High osmolarity activates the HOG MAPK signaling pathway in yeast *S. cerevisiae* which induces adaptive responses to hyperosmotic stress, including global readjustment of gene expression, transient cell cycle arrest, as well as accumulation of the compatible solute glycerol (Saito and Posas 2012).

6.1.1 HOG pathway architecture

The HOG signaling cascade is activated via two functionally redundant, but mechanistically distinct, Sln1 and Sho1 branches. Signals originating from either branch converge on the Pbs2 MAPKK, which is the activator of the Hog1 MAPK (Brewster et al. 1993; Maeda et al. 1994; Maeda et al. 1995). Either of the Sln1 or Sho1 branch is sufficient to cope with osmotic stress. However, the Sln1 branch holds a more prominent role in osmoadaptation as it is more sensitive to relatively small osmotic changes (O'Rourke and Herskowitz 2004; Hohmann 2009).

The Sho1 branch is controlled by Msb2 and Hkr1, which are two mucin-like transmembrane sensors (de Nadal et al. 2007; Tatebayashi et al. 2007) and are stimulated by hyperosmotic shock. This stimulation in turn prompts the Ste20 and Cla4 kinases to bind to the membrane-bound small G-protein Cdc42 and become activated (Lamson et al. 2002). Activated Ste20/Cla4 phosphorylates and activates the Ste11 MAPKKK (Raitt et al. 2000) which subsequently phosphorylates and activates the Pbs2 MAPKK that is associated with the Sho1 membrane protein (Maeda et al. 1995; Tatebayashi et al. 2006).

Both the Cdc42-Ste20 and the Sho1-Pbs2 complexes are localized at the membrane, hence Ste50 forms a complex with the Ste11 MAPKKK and mediates its membrane localization (Posas et al. 1998; Wu et al. 1999); first through association with the Opy2 anchor protein (Ekiel et al. 2009; Yamamoto et al. 2010) and then by interactions with Ste50-Cdc42 and Ste50-Sho1 (Truckses et al. 2006; Yamamoto et al. 2010). Although many important components of Sho1 branch are known, the knowledge about the activation mechanism is still vague. A unifying mechanism that ties together all the separate factors is yet to be presented.

The Sln1 branch is a variation of the "two-component system", controlled by a phospho-relay system which consists of the plasma membrane sensor and histidine kinase Sln1, the signal transmitter protein Ypd1, and the response regulator Ssk1 (Posas et al. 1996) . Sln1 is active under ambient conditions and inactive upon hyperosmotic shock (Maeda et al. 1994; Fassler and West 2010). Sln1 appears to sense the decreasing turgor pressure against the cell wall caused by cell shrinkage under hyperosmotic shock (Tamas et al. 2000; Schaber et al. 2010). On the other hand, Sln1 histidine kinase activity is enhanced by increased turgor pressure due to elevated intracellular glycerol concentration (Tao et al. 1999). Under iso-osmotic conditions, Sln1 autophosphorylates itself on a histidine residue, then the phosphate group is transferred to an aspartate residue in Sln1, and subsequently to a histidine group of Ypd1, and finally to an aspartate group of Ssk1 (Posas et al. 1996). The transfer of the phosphate group from Ypd1 to Ssk1 is very rapid and irreversible, which is consistent with the fact that Ssk1 is constitutively phosphorylated under normal osmotic conditions (Janiak-Spens et al. 2005). Phosphorylated Ssk1 is unable to bind Ssk2 and Ssk22 MAPKKK. Upon hyperosmotic conditions Ssk1 is unphosphorylated and binds to the regulatory domain of Ssk2 and Ssk22. This leads to autophosphorylation of the MAPKKK Ssk2 and Ssk22 and, therefore, phosphorylation of Pbs2 (Posas and Saito 1998).

The Sho1 and Sln1 branches converge on Pbs2 MAPKK by phosphorylation of the Ser514 and Thr518 residues through any of the MAPKKKs Ssk2/Ssk22 and Ste11 (Posas and Saito 1997). Phosphorylated Pbs2 activates Hog1 MAPK by dual phosphorylation of phosphorylation sites conserved among other MAPKs; Thr174 and Tyr176 (Brewster et al. 1993). Hog1 phosphorylation occurs rapidly but transiently upon hyperosmotic shock (Reiser et al. 1999). However, in severe osmotic stress, Hog1 phosphorylation is sustained for longer periods (Van Wuytswinkel et al. 2000). Hog1 is evenly distributed in

the cytoplasm and nucleus in normal condition. Hyperosmotic stress leads to rapid accumulation of Hog1 in the nucleus (Ferrigno et al. 1998; Reiser et al. 1999). Hog1 mutations at either Thr174 or Tyr176 prevent Hog1 translocation into the nucleus (Ferrigno et al. 1998; Reiser et al. 1999). It has been observed that severe osmotic shock leads to prolonged phosphorylation of Hog1 and a delayed induction of stress-responsive genes (Van Wuytswinkel et al. 2000; Hohmann 2002b). It has also been known for some time that Hog1 signaling and nuclear accumulation are delayed at higher stress levels (Mattison and Ota 2000; Van Wuytswinkel et al. 2000; Muzzey et al. 2009). In parallel to the Hersen group, we have shown that the delayed nuclear accumulation encompass Hog1, Msn2 (Paper I and (Miermont et al. 2013)), Mig1, Yap1 and Crz1 nuclear localization, as well as vesicular trafficking (Miermont et al. 2013). We found that the timing of Hog1 nuclear accumulation correlates with the degree of cells shrinkage and the cellular volume recovery rate (Paper I, Figure 2). Furthermore, we have shown that the general diffusion rate of Hog1 in the cytoplasm is dramatically reduced following severe volume reduction (Paper I, Figure 5). Our and Miermont's data suggest that higher level of osmostress causes cell volume compression below a threshold where molecular crowding may delay signal progression.

6.1.2 Transcriptional response

High osmolarity stress has a major effect on remodeling of genome expression (Gasch et al. 2000; Posas et al. 2000; Rep et al. 2000; Siderius et al. 2000; Causton et al. 2001; Yale and Bohnert 2001; de Nadal et al. 2011). It has been shown that gene expression is required for long-term adaptation to high osmolarity, since a number of mutants in the transcriptional machinery cause osmosensitivity (De Nadal et al. 2004; Zapater et al. 2007; Mas et al. 2009). There are a large number of genes whose transcription is induced in response to osmostress. However, only some of these genes respond to osmostress specifically, whereas the others respond to different types of stresses such as DNA damage, heat shock, osmostress, or oxidative stress (Gasch et al. 2000; Capaldi et al. 2008). On the other hand, products of genes that are down-regulated under stress are involved in protein synthesis and in growth-related processes (Gasch 2007; Martinez-Montanes et al. 2010).

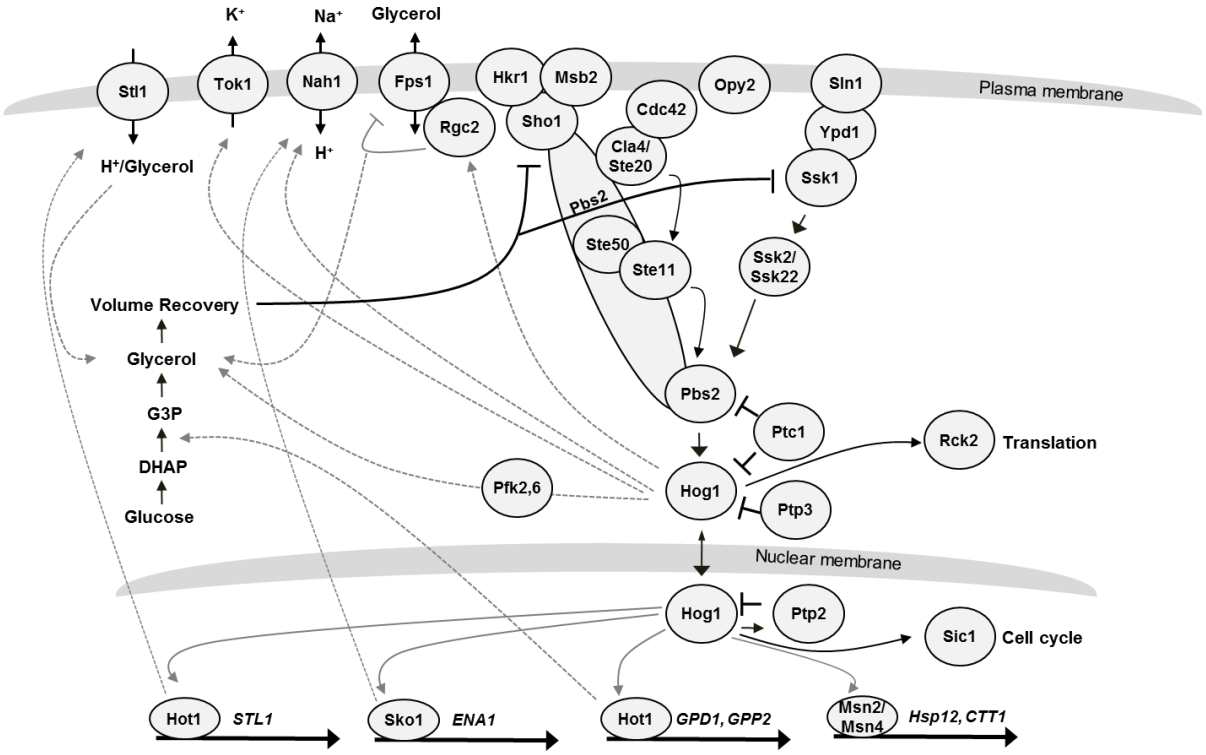

Figure 4. Overview of the yeast HOG pathway.

Upon hyperosmotic shock, Sho1 and Sln1 branches phosphorylate the Pbs2 MAPKK which in turn phosphorylates and activates the Hog1 MAPK. Phosphorylated Hog1 migrates into the nucleus and associates with different DNA-binding proteins to mediate transcriptional regulation. Hog1 also coordinates cytoplasmic osmoresponses such as control of glycerol transport, ionic fluxes, metabolic enzymes, and protein translation.

Phosphorylated Hog1 accumulates in the nucleus where it controls gene expression in collaboration with DNA-binding proteins such as Hot1, Msn2/4, and Sko1 (Schuller et al. 1994; Rep et al. 1999) which affect the expression of hundreds of genes (Gasch et al. 2000; Posas et al. 2000; Rep et al. 2000; Capaldi et al. 2008; de Nadal and Posas 2010; Martinez-Montanes et al. 2010). Direct phosphorylation is one of the mechanisms by which Hog1 controls initiation of transcription, for example phosphorylation of Sko1 by Hog1 (Proft et al. 2001). However, phosphorylation is not required for regulation of a number of transcription factors such as Hot1 (Alepuz et al. 2003). Hog1 interacts with the RNA Pol II and with general components of the transcription machinery (Alepuz et al. 2003) and also with the chromatin structure remodeling (RSC) complex and recruits it to coding regions of osmo-responsive genes (Mas et al. 2009). Transcription factors can act individually or in co-association with other factors to coordinate a dynamic biological response (Ni et al. 2009).

Hot1 is the key transcription factor controlling glycerol production and uptake under osmotic stress (Rep et al. 1999; Rep et al. 2000). It is involved in the control of expression of *GPD1* and *GPP2,* which encode the enzymes that convert glyceraldehyde-3-phosphate to glycerol (Larsson et al. 1993; Albertyn et al. 1994a). Msn2 and Msn4 are partly redundant transcription factors necessary for transcription of many stress-induced genes, including those related to osmotic stress (Martinez-Pastor and Estruch 1996; Schmitt and McEntee 1996). Sko1 controls the expression of several regulators of the osmo-stress response, such as *ENA1,* which encodes a plasma membrane Na^+ export pump (Proft and Serrano 1999), and *GRE2,* which encodes an enzyme involved in ergosterol metabolism (Warringer and Blomberg 2006).

6.1.3 Cytosolic targets

Hyperosmotic stress leads to rapid phosphorylation and nuclear localization of Hog1, where Hog1 triggers transcriptional responses. However, cells in which Hog1 cannot enter the nucleus- either due to lack of Nmd5 or a tethering of Hog1 to the plasma membrane- are still osmoresistant. This suggests that the activation of gene expression is not critical for osmoadaptation (Westfall et al. 2008).

Therefore, it appears that cytoplasmic events mediated by activation of Hog1 may be sufficient to deal with osmotic stress. Hog1 has various cytosolic targets. For instance, Nha1, a Na^+/H^+ anti-porter and the Tok1 potassium channel, which are located at the plasma membrane, are activated by Hog1-dependent phosphorylation upon osmotic stress (Proft and Struhl 2004). Moreover, Hog1 interacts with the Ser/Thr kinase Rck2, which acts downstream of Hog1 and controls a subset of the responses induced upon osmotic stress. Overexpression of *RCK2* suppresses the osmosensitivity of *hog1*Δ mutant. (Bilsland-Marchesan et al. 2000). Furthermore, Hog1 phosphorylates the plasma membrane protein Fps1, an aquaglyceroporin, upon arsenite and acetic acid stresses (Thorsen et al. 2006; Mollapour and Piper 2007). An increase in medium osmolarity results in a rapid increase of cellular glycerol levels in order to adapt intracellular osmotic pressure. A link between HOG pathway and Pfk26, which is an activator of glycolysis, leads to stabilizing glycolytic flux (Petelenz-Kurdziel et al. 2013). It has been demonstrated that the activity of Pfk26 is decreased in absence of Hog1 (Dihazi et al. 2004; Bouwman et al. 2011).

Hog1 regulates the stability of many mRNAs whose levels change upon osmotic stress (Molin et al. 2009; Romero-Santacreu et al. 2009; Miller et al. 2011).

6.1.4 Glycerol accumulation

Glycerol is produced from the glycolytic intermediate dihydroxyacetone phosphate (DHAP) through a two steps process. First, DHAP is converted to glycerol-3-phosphate (G3P) by the NAD-dependent glycerol-3-phosphate dehydrogenases, Gpd1 and Gpd2 (Ansell et al. 1997) Then, G3P is dephosphorylated to glycerol by the glycerol-3-phosphatases Gpp1 and Gpp2 (Norbeck et al. 1996; Påhlman et al. 2001). The double mutants, *gpd1Δ gpd2Δ* and *gpp1Δ gpp2Δ*, are osmosensitive due to inability to produce glycerol (Siderius et al. 2000; Klipp et al. 2005). Glycerol accumulation is partially controlled via a regulated, Fps1-mediated export of glycerol (Petelenz-Kurdziel et al. 2013). Fps1 closure upon hyperosmotic stress ensures accumulation of glycerol and consequently osmoadaptation (Tamas et al. 1999).

Control of glycerol accumulation is probably the most important role of the HOG pathway in osmotic adaptation (Hohmann 2002b; Yancey 2005). In fact in Paper II, we describe essential roles of Hog1 using engineered yeast cells, in which osmoadaptation was reconstituted in a Hog1-independent manner by rewiring osmostress signaling through the Fus3/Kss1 MAPKs. Fus3 and Kss1 are improperly activated via crosstalk in *hog1Δ* cells upon osmostress (O'Rourke and Herskowitz 1998; Davenport et al. 1999). Our data suggests that osmotic up-regulation of the two Hog1-dependent glycerol biosynthesis genes, *GPD1* and *GPP2*, is sufficient for successful synthetic osmoadaptation (Paper II, Figure 2).

Glycerol production and accumulation is controlled at different levels and Hog1 appears to play a role in all those mechanisms. This includes control of expression of the Stl1 active glycerol uptake system (Ferreira et al. 2005), control of the activity of the glycerol export channel Fps1 (Lee et al. 2013), control of expression of genes encoding enzymes in glycerol production (Albertyn et al. 1994a; Eriksson et al. 1995), and control of glycolytic flux via altering Pfk26 activity (Dihazi et al. 2004; Petelenz-Kurdziel et al. 2013).

Glycerol is produced even under iso-osmotic conditions in order to maintain the redox balance and this process is essential under anaerobic conditions (Ansell et al. 1997).

6.1.5 Feedback control of HOG pathway

Constitutive Hog1 phosphorylation is lethal (Maeda et al. 1993) due to the inhibitory effect of active Hog1 on cell cycle progression (Clotet and Posas 2007). Therefore, Hog1 activity is tightly regulated.

Experimental results obtained from various mutants with decreased and increased ability to accumulate glycerol have shown that accumulation of the compatible solute glycerol is the most important negative feedback mechanism on Hog1 pathway signaling (Brewster et al. 1993; Albertyn et al. 1994b). Mathematical modeling and simulation of the feedback control system suggested that cellular adaptation to the new conditions controls the period of Hog1 activation (Klipp et al. 2005; Muzzey et al. 2009) which is consistent with experimental results. Nevertheless, transcriptional induction of genes needed for glycerol accumulation is not fast enough to be considered as a rapid down-regulator of Hog1 activity (Hirayama et al. 1995). However, Hog1 regulates glycerol accumulation through closing of the glycerol channel Fps1 (Beese et al. 2009; Lee et al. 2013), also activation of 6-phosphofructo-2-kinase (Dihazi et al. 2004; Klipp et al. 2005; Bouwman et al. 2011) and, indirectly, activation of the Gpd1 protein (Lee et al. 2012; Oliveira et al. 2012).

In addition, Hog1 kinase activity must also be controlled by dephosphorylation of Thr174 and Tyr176. The phosphorylation state of Hog1 is controlled by different phosphatases, including two tyrosine phosphatases Ptp2 and Ptp3 (Jacoby et al. 1997; Wurgler-Murphy et al. 1997), as well as three serine/threonine phosphatases Ptc1, Ptc2, and Ptc3 (Warmka et al. 2001). Ptp2 and Ptp3 dephosphorylate Tyr176 (Jacoby et al. 1997; Wurgler-Murphy et al. 1997) in the nucleus and cytoplasm, respectively (Mattison and Ota 2000). The serine/threonine phosphatases dephosphorylate Thr174. Ptc1 has the most important role for dephosphorylation of Hog1 among serine/threonine phosphatases (Warmka et al. 2001). Overexpression of any of these phosphatases prevents the lethal effects of inappropriate activation of the HOG pathway (Jacoby et al. 1997; Wurgler-Murphy et al. 1997; Warmka et al. 2001). Simultaneous deletion of *PTC1* and *PTP2* is lethal because it causes constitutive Hog1 phosphorylation (Maeda et al. 1993).

Active Hog1 phosphorylates upstream components, and hence down-regulates the HOG pathway. One such target is Ste50 (Hao et al. 2008). As mentioned, interaction of Ste50 and Opy2 is needed for Hog1 activation through the SHO1 branch (Ekiel et al. 2009;

Yamamoto et al. 2010), whereas phosphorylation of Ste50 by Hog1 reduces its affinity for interacting with Opy2 and hence acts as negative feedback mechanism (Yamamoto et al. 2010).

6.1.6 Crosstalk between HOG pathway and other MAPK pathways

Since MAPK pathways share protein kinases and phosphatases, there are several nodes for interaction between them. Interaction between pathways may have evolved for different reasons, such as to integrate signals, to produce a variety of responses to a signal, and to reuse proteins between pathways. Therefore, prevention of one MAPK pathway from adventitious activation by parallel pathways seems to be crucial (Hall et al. 1996).

Osmotic stress in *hog1*Δ causes an increase in Fus3 phosphorylation, activation of pheromone reporter gene *FUS1*, and increased sensitivity to growth arrest by pheromone. Hence, the HOG pathway represses mating pathway activity (Hall et al. 1996). Moreover, the mating deficiency of *ste4*Δ and *ste5*Δ, the activator and the scaffold protein in the pheromone pathway, is partially suppressed in the *hog1*Δ mutant in the presence of 1 M sorbitol (O'Rourke and Herskowitz 1998). Furthermore, Kss1, the MAP kinase of the pseudohyphal development pathway, becomes activated by osmotic stress in a mutant with a partially functional allele of *PBS2*, *pbs2-3* (Davenport et al. 1999).

Although these types of crosstalk occur in mutants, there also seems to be some crosstalk upon osmotic shock in wild type cells. For instance, phosphorylation of Slt2, the MAPK of cell wall integrity pathway, is transiently stimulated after activation of HOG pathway. Apparently, Slt2 phosphorylation is related to changes in the glycerol turnover and its activation is mostly dependent on one of the sensors of the pathway, Mid2 (Garcia-Rodriguez et al. 2005).

Although the crosstalk between MAPK signaling pathways has been intensely investigated, the mechanisms that control crosstalk are still incompletely understood.

6.1.7 Hog1 activation causes transient cell cycle arrest

Different stress conditions, such as heat stress, DNA damage, and hyper osmolarity, affect proliferation, therefore the cells must control the cell cycle under these stress

conditions in order to prevent damage and allow appropriate cellular adaptation (Flattery-O'Brien and Dawes 1998; Li and Cai 1999; Wang et al. 2000; Alexander et al. 2001).

The cell cycle delay caused by activated Hog1 occurs at different levels of cell cycle control and enables cells to develop osmo-adaptive responses before cell cycle progression resumes (Clotet and Posas 2007; Yaakov et al. 2009). The length of the cell cycle delay depends on the degree of the stress (Adrover et al. 2011), and prolonged Hog1 activation leads to cell death (Vendrell et al. 2011).

It has been demonstrated by experimental approaches and mathematical modeling that activation of Hog1 causes a cell cycle delay in G_1 phase via direct phosphorylation of Sic1, a cyclin dependent kinase (CDK) inhibitor, as well as inhibition of transcription of the genes encoding the G_1 cycling Cln1 and Cln2 (Belli et al. 2001; Escote et al. 2004; Zapater et al. 2005; Adrover et al. 2011). In stressed S-phase cells, Hog1 promotes S phase delay by down-regulating the S-phase cyclins Clb5 and Clb6, and also by interacting with various proteins of the replication complexes and postponing phosphorylation of the Dpb2 subunit of the DNA polymerase (Adrover et al. 2011). Hog1 prevents G_2 phase progression by decreasing kinase activity of cyclin/CDK complex, Clb2/Cdc28, in addition to down-regulating Clb2 (Alexander et al. 2001; Clotet et al. 2006). Moreover, upon osmotic stress, Hog1 promotes the exit from mitosis (Reiser et al. 2006).

6.2 Cell wall integrity pathway (CWI)

Several conditions stress the structure and function of the yeast cell wall: hypotonic medium, heat shock, treatment of cells with glucanases, exposure to chitin-binding agents, oxidative stress, depolarization of the actin cytoskeleton, and pheromone-induced morphogenesis all stimulate cell wall integrity pathway (Harrison et al. 2004; Levin 2005). Stretching of the plasma membrane and alterations of its connections to the cell wall seem to be the common effect caused by all of these conditions (Chen and Thorner 2007). Activation of CWI signaling regulates the production of various carbohydrate polymers of the cell wall (glucan, mannan, and chitin), as well as their polarized delivery to the site of cell wall remodeling (Levin 2011).

6.2.1 CWI pathway architecture

The CWI pathway activation occurs through cell surface sensors Wsc1, Wsc2, Wsc3, Mid2, and Mtl1. They are all mucin-like plasma membrane proteins with similar structure including short C-terminal cytoplasmic domains, a single transmembrane domain, as well as a highly glycosylated serine/threonine-rich periplasmic ectodomain (Hohmann 2002b; Levin 2011). Wsc1 and Mid2 are the most important of these sensors. The *wsc1*Δ is unable to activate the Slt2 MAPK at high temperature (Gray et al. 1997; Verna et al. 1997) and the *mid2*Δ mutant dies following pheromone treatment (MID: mating pheromone-induced death) (Ketela et al. 1999; Rajavel et al. 1999).

Wsc1 and Mid2 interact with the N-terminal domain of the Rom1/2 Guanine Exchange Factors (GEFs) through their cytoplasmic domains and stimulate nucleotide exchange on the small G-protein Rho1 (Philip and Levin 2001). In the GTP-bound state, Rho1 activates Pkc1 which is the main effector of Rho1. Activation of Pkc1 in turn triggers activation of the CWI MAPK cascade that includes the MAPKKK Bck1, the MAPKKs Mkk1 and Mkk2 and the MAPK Slt2 (Levin et al. 1990; Lee and Levin 1992; Irie et al. 1993; Lee et al. 1993).

6.2.2 Effectors downstream of CWI pathway

The CWI pathway mediates transcriptional responses via two regulators; Rlm1 and the SBF complex (Baudouin et al. 1999). Genome-wide studies have revealed that Rlm1 regulates the expression of at least 25 genes, most of which encode cell wall proteins or are involved in cell wall biogenesis (Jung and Levin 1999). The SBF complex is a dimer-composed of Swi4 and Swi6- and a regulator of G_1-specific transcription. In response to cell wall stress, SBF regulates gene expression in a manner that is independent of its role in G_1-specific transcription (Kim et al. 2008; Truman et al. 2009). In addition to Rlm1 and the SBF complex, the Msn2, Msn4, Hsf1, and Skn7 transcription factors are involved in cell wall stress responses (Li et al. 1998; Jung and Levin 1999; Garcia et al. 2004).

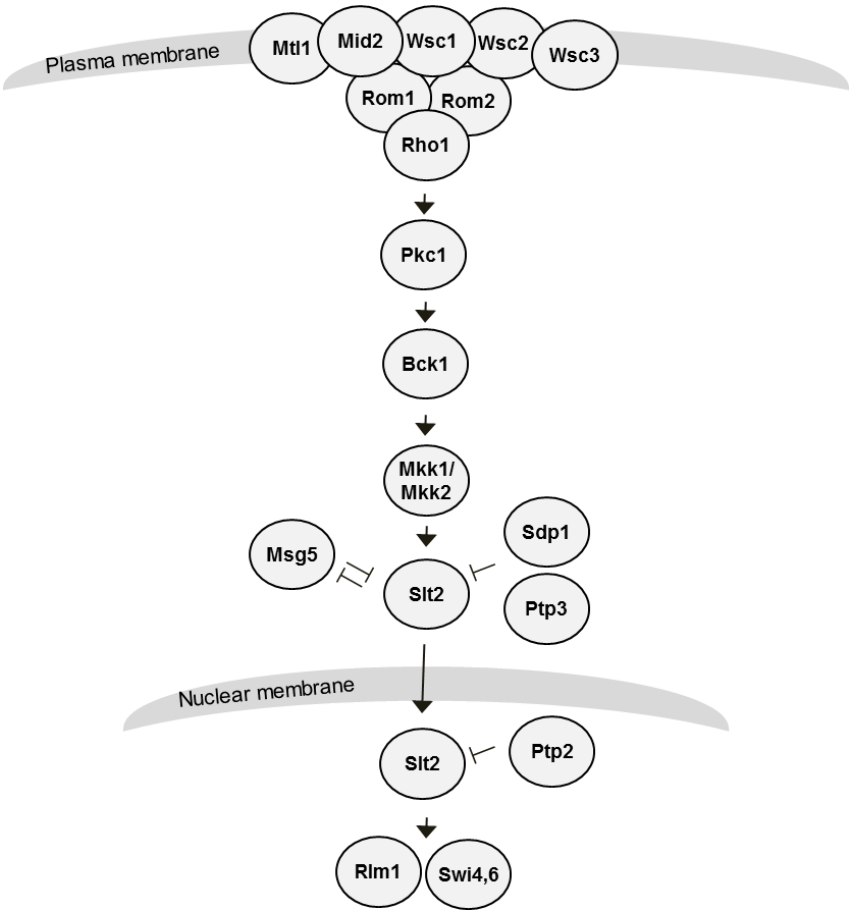

Figure 5. Overview of yeast CWI pathway.

The cell surface sensors activate Rho1 and the downstream Pkc1-activated MAPK cascade: MAPKKK Bck1, MAPKK Mkk1 and Mkk2 and MAPK Slt2. Two transcription factors, Rlm1 and SBF complex, are nuclear targets of Slt2.

The growth defects displayed by the *rlm1*Δ *swi4*Δ double mutant are less severe compared to those of the *slt2*Δ mutant, and the double mutant also does not seem to have defects in actin polarization. This suggests that in addition to transcriptional responses, Slt2 also has cytoplasmic targets (Levin 2005). Cytoplasmic roles of CWI include regulation of the actin cytoskeleton and stress-induced re-localization of chitin synthase III (Chs3), which is required for synthesis of chitin to strengthen the cell wall (Valdivia and Schekman 2003) and activation of the Cch1/Mid1 Ca^{2+} channel (Bonilla and Cunningham 2003).

6.2.3 Down-regulation of signaling: MAPK phosphatase

Slt2 activity is down-regulated by four protein phosphatase including the Ptp2 and Ptp3 tyrosine phosphatases and the dual specificity (Tyr and Ser/Thr) paralogs Sdp1 and Msg5

(Levin 2011). Among these phosphatases, Sdp1 is the only one to target Slt2 specifically and its role is to down-regulate Slt2 after stimulation to re-establish the basal state (Collister et al. 2002). In contrast to Sdp1, Msg5 is responsible for maintaining a low basal activity of Slt2 in the absence of stress (Marin et al. 2009). Deletion of Msg5 results in an increased basal level of Slt2 phosphorylation. However, increased Slt2 phosphorylation is not accompanied by an increase in its protein kinase activity. This suggests that phosphorylation is necessary but not sufficient for Slt2 protein kinase activity (Marin et al. 2009). The tyrosine phosphatases Ptp2 and Ptp3 dephosphorylate Slt2 as well as Fus3 and Hog1 MAPKs. However, Ptp2 appears to be more effective than Ptp3 in down-regulation of Slt2 (Mattison et al. 1999).

6.2.4 Hypo osmotic shock triggers CWI pathway

Hypo osmotic shock induces a rapid, but transient, activation of CWI signaling (Davenport et al. 1995; Kamada et al. 1995). Slt2 activation occurs within 15 seconds after an osmotic downshift. To maintain proper turgor pressure and prevent bursting, cells release glycerol through the Fps1 membrane protein (Tamas et al. 1999). In addition to the CWI pathway, hypo osmotic stress activates the Sln1 sensor, which results in inhibition of the HOG pathway and activation of the Skn7 transcription factor (Li et al. 1998; Hohmann 2002b). Skn7 interacts not only with the HOG pathway (Fassler et al. 1997; Ketela et al. 1998) but also with CWI pathway (Brown et al. 1994; Alberts et al. 1998). Skn7 appears to have many different roles, such as controlling cellular responses to cell swelling and hypo osmotic signals, control of cell proliferation, and stress responses (Li et al. 1998; Bouquin et al. 1999; Tao et al. 1999; Hohmann 2002b; Li et al. 2002). Although Skn7 is involved in so many different cellular processes, it is not an essential protein. This suggests that Skn7 participates in modulating responses and/or it is involved in redundant systems (Hohmann 2002b). It seems that Skn7 functions opposite to HOG pathway and in parallel to CWI pathway, which respond to high and low osmolarity respectively. Hence, Skn7 may integrate input from HOG and CWI osmosensing pathways (Hohmann 2002b).

6.2.5 The CWI pathway and its role in arsenite tolerance

We have shown in Paper IV that Slt2 is phosphorylated upon arsenite exposure (Paper IV, Figure 4B). Deletion of *WSC1*, *BCK1*, and *SLT2* caused arsenite sensitivity (Paper IV, Figure 1), which suggests that the CWI pathway contributes to arsenite tolerance.

Furthermore, we observed that *SLT2* and *FPS1* overexpression overcomes the sensitivity of the *slt2Δ* mutant upon arsenite exposure. However, *bck1Δ* sensitivity is not rescued upon simultaneous overexpression of *SLT2* and *FPS1* (Paper IV, Figure 3A). In addition, a kinase-dead version of Slt2 (Flandez et al. 2004) could not complement the As(III) sensitivity of the *slt2Δ* mutant (Paper IV, Figure 3B). Taken together, both the activation of Slt2 via Bck1 and the kinase activity of Slt2 are required for arsenite tolerance.

7 The Fps1 glycerol transporter: Balancing between Hog1 and Slt2 regulation

The HOG pathway and the CWI are stimulated upon hyperosmotic and hypo osmotic shock, respectively (Brewster et al. 1993; Davenport et al. 1995). Although it seems that these two pathways have opposite functions, in fact, they collaborate in the same process (Hohmann 2002b). HOG pathway activation results in increased turgor pressure, which is inferred as a stimulus by the cell integrity pathway and results in coordination of cell wall strength and cell expansion and consequently decreased turgor pressure. Therefore, these two pathways regulate osmotic homeostasis and an appropriate turgor pressure for cell morphogenesis. For instance, during cell growth, activation and deactivation of both pathways within short intervals may provide the balance between cell expansion and cell wall development (Hohmann 2002b).

The yeast *S. cerevisiae* adapts to hyperosmotic shock by accumulation of glycerol, while hypo osmotic shock results in a rapid glycerol efflux to prevent cells from bursting. The yeast plasma membrane is impermeable for glycerol and therefore the cell requires a transport protein to control passive glycerol diffusion (Oliveira et al. 2003) as well as glycerol export upon hyperosmotic stress (Tamas et al. 1999). These functions are mediated by Fps1, which might be a link between the HOG and CWI pathways to control glycerol flux during basal conditions and upon osmostress.

7.1 Fps1 regulation by the HOG pathway

Fps1 mediates bidirectional transport of glycerol across the plasma membrane. However, its major role seems to be to mediate osmostress-induced glycerol export (Luyten et al. 1995; Tamas et al. 1999). In the absence of osmotic stress, the regulator of the glycerol channel-2 (Rgc2) binds to the C-terminal cytoplasmic domain of Fps1 and maintains it in the open channel state to allow glycerol efflux. After a hyperosmotic shock, Fps1 is rapidly inactivated (closed) to preserve the glycerol produced by the cell (Tamas et al. 1999). Active Hog1 phosphorylates Rgc2, which mediates rapid eviction of Rgc2 from Fps1 and channel closure (Lee et al. 2013). The *fps1Δ* mutant hyper accumulates glycerol

under hyperosmotic stress. At the same time the *fps1Δ* mutant can adapt to hyperosmotic shock similar to wild type cells (Tamas et al. 1999). Nevertheless, Fps1 regulation is crucial since cells that express a constitutively open Fps1 version do not accumulate glycerol as efficiently as wild type and grow poorly in high osmolarity conditions (Tamas et al. 1999).

In addition to hyperosmotic shock, Hog1 directly regulates Fps1 transport activity in response to arsenite exposure (Thorsen et al. 2006) as well as Fps1 protein stability in response to acetic acid treatment (Mollapour and Piper 2007).

7.2 Fps1 regulation by CWI pathway

A hypo osmotic shock activates Fps1 to mediate glycerol efflux (Luyten et al. 1995; Tamas et al. 1999). The Slt2 MAPK is also activated upon hypo osmotic stress which makes it a candidate for activating Fps1. Pheromone treatment of yeast cells causes Slt2 activation, which is needed for formation of mating projections. When cells are first adapted to high external osmolarity and then treated with pheromone, Hog1 becomes activated in an Slt2- and Fps1-dependent manner. These observations are consistent with the idea that Slt2 mediates Fps1-dependent glycerol release, which in turn invokes Hog1 activation to counteract high external osmolarity (Baltanas et al. 2013). While these findings provide strong evidences for Fps1 regulation by Slt2, the molecular mechanism of this regulation is still unknown.

We have shown that Slt2 MAPK affects the Fps1 arsenite transport activity (Paper IV, Figure 6). Unlike in wild type cells, overexpression of Fps1 does not suppress the arsenite sensitivity of the *slt2Δ* mutant (Paper IV, Figure 2).

7.3 Analysis of hyper/hypo osmotic stress responses

In Paper III we studied the mechanisms of a hypothetical crosstalk between the HOG and the CWI pathway by analyzing Hog1 and Slt2 phosphorylation dynamics. We first treated cells with a hyperosmotic shock (0.8 M sorbitol), and then decreased sorbitol concentration again to 0.27 M (Paper III, Figure 1B, C). When the hypo osmotic shock is

applied at a certain time point following hyperosmotic stress, it causes volume increase to a level above the initial volume. This scenario is hence equivalent to a hypo osmotic shock and the internal glycerol levels need to be adjusted to restore the appropriate cell volume (Paper III, Figure 1C). We implemented a family of simple mathematical models representing different hypotheses about possible crosstalk mechanisms, which were formulated in the literature (Schaber et al. 2012; Schaber et al. 2014). These mathematical models constituted combinations of ordinary differential equations (ODEs) and algebraic equations. We measured cell volume recovery (Paper III, Figure 4C) and intracellular glycerol (Paper III, Figure 5C) for hyperosmotic shock conditions to fit the model parameters. We ranked the models according to the Akaike Information Criterion corrected for small samples (AICc).

We started with models in which both the HOG and CWI pathways employed a fixed activation threshold (Paper III, Figure 1D, Figure 2; models without v14, v15 and v16) and we showed that all models describe Hog1 phosphorylation dynamics very well. However, none of the tested models were able to reproduce Slt2 activation dynamics when we shifted cells from hyperosmotic to hypo osmotic after 4 min. Therefore, we concluded that the mechanism of Slt2 activation must be different from Hog1 activation.

Then, we assumed that the volume activation threshold for Slt2 is not fixed but is regulated by a sensitizing mechanism. We found that with the sensitized Slt2 activation scenario, the best-ranked model can reproduce Slt2 activation of all experimental data. In order to validate this model, we monitored Hog1 and Slt2 phosphorylation in response to hyperosmotic shock (0.4M and 0.8M) followed by hypo osmotic shock (0.27M) in the constitutively open *Fps1-Δ1* mutant (Paper III, Figure 6 and 7). This experiment confirmed that the data supports the sensitized Slt2 activation model which means that the volume activation threshold for Slt2 is not fixed. Instead, the threshold is adaptive in a way that is decreases with decreasing volume. The CWI pathway appeared to sense turgor pressure by monitoring the plasma membrane and its biophysical connections to the cell wall, both of which may passively adapt to volume changes rather quickly. However, volume shifts into the other direction may trigger CWI. Therefore, the sensitizer may be the biophysical flexibility inherent to the cell wall and the plasma membrane.

It has previously been demonstrated that Slt2 activation mediates glycerol release through Fps1 (Baltanas et al. 2013). However, our best-ranked model could not support this mechanism (Paper III, Table1). Nevertheless, our third model is able to reproduce and predict experimental data regarding Fps1 regulation by Slt2. This suggests that, although our best-ranked model does not support the Slt2 role in Fps1 regulation, it cannot be totally rejected. In fact, we have shown that in the model in which Fps1 regulation by Slt2 is supported, the role of Hog1 on Fps1 regulation is much stronger. Therefore we suggested that HOG pathway controls Fps1 regulation for both hyper and hypo osmotic stress.

8 Osmoadaptation in medium with non-fermentable carbon source

Cellular adaptation to altered nutrient availability and osmotic shock are linked since nutrient deprivation itself is a stressful condition. Nutrient restriction stimulates responses that lead to the remodeling of cellular metabolism, which is mediated by several signaling systems with partially redundant functions (Gasch et al. 2000; Causton et al. 2001; Hohmann 2002b). For instance the Snf3/Rgt2 glucose-sensing pathway, the Gpr1-cAMP-protein kinase A pathway, the Snf1 pathway, and the TOR pathway regulate proper responses to the availability or quality of carbon and energy sources (Hohmann 2002b).

In order to investigate how different carbon sources affect osmoadaptation and HOG pathway regulation, we studied phosphorylation and nuclear localization of Hog1, as well as volume recovery of cells growing in medium with ethanol as carbon source. We further measured gene expression of several osmo-induced genes in response to hyperosmotic shock in ethanol-grown cells. In particular we aimed at addressing two issues: (1) how the activity of different metabolic pathways is integrated when cells cope with osmotic stress and (2) whether the osmolyte system differs in ethanol-grown cells.

8.1 HOG pathway activation in ethanol medium

Growth assays in the presence of 400 mM NaCl showed that the *hog1*Δ mutant was sensitive to osmostress on ethanol and glucose medium (Paper V, Figure 1C), which suggests that HOG pathway activation is required for osmoadaptation of ethanol-grown cells. Therefore, to determine the quantitative characteristics of HOG pathway regulation in ethanol medium, we investigated phosphorylation and nuclear translocation of Hog1 as well as cell volume recovery under hyperosmotic stress.

Similar to glucose medium, both phosphorylation and nuclear residence levels of Hog1 increased following osmotic shock in ethanol-grown cells (Paper V, Figure 2A, B, and C). However, the amount of nuclear Hog1 appears to be much lower in ethanol-grown cells, which is consistent with the fact that total level of Hog1 is also much lower in ethanol medium.

Next, we determined the volume recovery profile of ethanol-grown cells after osmotic shock and showed that volume recovery starts around the same time point for glucose- and for ethanol-grown cells. However, volume recovery is much slower in ethanol-grown cells. As discussed in paper I, Hog1 nuclear accumulation correlates with cell volume recovery dynamics. Ethanol-grown cells demonstrate the same pattern (Paper V, Figure 2C and D).

8.2 Trehalose versus glycerol

Glycerol production is an essential event in osmoadaptation and it is controlled by the HOG pathway (Albertyn et al. 1994b; Ansell et al. 1997). However, in Paper V, we found that the production of glycerol is not enhanced during osmotic stress in ethanol-grown cells (Paper V, Figure1A). This suggests that ethanol-grown cells accumulate other osmolytes to deal with hyperosmotic shock.

Initially, trehalose was considered as just a reserve metabolite. However, trehalose has been implicated as a potential stress protectant that accumulates in *S. cerevisiae* during various stress conditions, including hyperosmotic stress (Nwaka and Holzer 1998; Singer and Lindquist 1998; Thevelein et al. 2000; Francois and Parrou 2001; Elbein et al. 2003). Trehalose enables the cells to dispose of bound water and replace the water around biomolecules (Crowe et al. 1984; Sano et al. 1999). Furthermore, it has been shown that trehalose has a role as osmolyte in bacteria *Escherichia coli* (Larsen et al. 1987; Kempf and Bremer 1998).

Therefore, we have measured intracellular trehalose in response to hyperosmotic shock in ethanol-grown cells and observed that, unlike glycerol, trehalose concentration increased (Paper V, Figure1B). Ethanol-grown cells accumulated significantly more trehalose under osmostress than ethanol-grown cells.

8.3 Gene expression analysis

We also studied expression levels of five osmo-induced genes (*STL1*, *ALD2*, *GPD1*, *HSP12*, and *TPS1*) in wild type, *hog1*Δ, *bcy1*Δ *tpk1*Δ *tpk3*Δ *tpk2(att)*, and *msn2*Δ *msn4*Δ

mutants to initially explore how gene expression is remodeled in response to hyperosmotic shock in ethanol-grown cells and which pathways control those processes (Paper V, Figure 4). Except *GPD1*, the other four genes showed higher mRNA levels in ethanol-grown wild type cells as compared to glucose-grown cells. The expression level of all genes decreased dramatically in the *hog1*Δ background; however, it seemed that expression of the genes tested is less Hog1-dependent in ethanol medium. This suggests that another pathway may partially replace Hog1 in ethanol medium.

Therefore, we looked at gene expression level in the attenuated PKA mutation and the *msn2*Δ *msn4*Δ double mutant. The expression level of all genes except *STL1* is decreased in the attenuated PKA. However, it does not seem that the PKA pathway has a significant role in up-regulation of osmo-stressed cells in ethanol medium. On the other hand, expression of *TPS1*, *ALD2* and *HSP12* is strongly dependent on Msn2 and Msn4. Nevertheless, expression of all these genes is still upregulated in both glucose and ethanol-grown cells in the *msn2*Δ *msn4*Δ mutant. In summary, we have shown that the carbon source affects the osmostress-induced gene expression pattern, that expression is controlled by the same signaling pathways but their relative importance may be shifted dependent on the carbon source.

9 Concluding remarks and future perspectives

Osmoregulation is an active process that controls the cellular water balance and maintains homeostatic conditions of a living cell. In the yeast *S. cerevisiae*, osmoregulatory responses are mainly executed and regulated by the high osmolarity glycerol (HOG) signaling system. The HOG pathway is activated following cell shrinkage, and coordinates a wide range of adaptive responses from gene expression in the nucleus to post-transcriptional process in the cytosol in order to produce and accumulate the compatible solute glycerol. Integrating traditional molecular approaches with new technologies for comprehensive and quantitative measurements, such as high throughput experiments and single cell analysis as well as synthetic biology and mathematical modeling, facilitates achieving an inclusive view of the dynamics of the underlying adaptation processes. In particular, computational simulations and parallel quantitative time course experimentation provide an opportunity to investigate how the interaction and kinetics of proteins affect the timing, magnitude, and period of signaling processes. This integration also appeared to be an informative approach to address the timeline of events and cross-regulation of signaling pathways.

In this thesis, several osmo-responsive events were investigated by applying single cell methods. The single cell data obtained from Hog1 nuclear translocation and cell volume recovery in microfluidics system revealed that the strength of osmotic stress affects the time-dependent profile of osmoadaptation. Possibly, under higher stress levels, yeast cells prioritize regulation of cytosolic targets such as glycerol metabolism and glycerol transport in the early stages of osmoadaptation. Moreover, the diffusion dynamics of cytosolic Hog1, as measured by Fluorescence Correlation Spectroscopy (FCS), is decreased following higher stress level and hence bigger cell volume loss. This data provided direct evidence that signaling processes are slowed down most probably due to molecular crowding caused by cell compression. Molecular crowing also affects proteins' movements and their interactions. Potentially, these alterations may serve as a stimulus for osmosensors of SLN1 branch. This branch seems to detect changes in the interaction between the plasma membrane and the cell.

We revealed essential roles of Hog1 by rewiring osmostress signaling through the MAPK network. The synthetic *hog1*Δ osmoadaptation strains were able to produce glycerol

under the control of the Fus3/Kss1-dependent *FUS1* promoter. Intracellular glycerol measurement and cell volume recovery data of synthetic osmoadaptation strains showed that rapid and efficient glycerol accumulation suppressed the osmosensitive phenotype of the *hog1*Δ mutant. Therefore, the data indicated that one of the essential osmoadaptation roles of Hog1 is to regulate genes involved in the biosynthesis of glycerol. Taken together, it appeared that the number of essential osmo-responsive genes regulated by Hog1 may be significantly lower than anticipated. Applying synthetic biology, further investigation could be performed to achieve novel and complementary knowledge about Hog1 roles in regulation of cell cycle, translation, and crosstalk. For instance, Hog1 mediates a transient cell cycle arrest during osmoadaptation. Fus3 also causes cell cycle arrest in the synthetic *hog1*Δ strains by regulation of cyclin dependent kinase inhibitor Far1. This may affect the growth of the synthetic *hog1*Δ strains. Therefore, introducing a negative feedback into the synthetic *hog1*Δ strains may suppress the osmosensitivity of cells and can determine the contribution of cell cycle arrest in osmoadaptation.

Hog1 mediates Fps1 closure upon hyperosmotic shock in order to facilitate rapid glycerol accumulation. However, growth assays, intracellular glycerol measurements, and volume recovery data from synthetic osmoadaptation strains revealed that although proper Fps1 closure is essential for osmoadaptation, Hog1 is dispensable for regulation of Fps1 gating. This suggests that other MAPK proteins may be involved in Fps1 gating. In fact, there is evidence that Slt2 mediates Fps1 regulation in response to pheromone induction (Baltanas et al. 2013) and arsenite exposure (Paper IV). Therefore, we combined mathematical modeling with experimental analysis of how HOG and CWI respond to external osmotic changes. Unexpectedly, our best-ranked model does not support that Slt2 regulates Fps1. However, in our experimental conditions, rapid deactivation of Hog1 upon a shift to hypo osmotic condition appeared to be sufficient to re-open Fps1channel again. Therefore the concept of a Hog1-Stl2 antagonism to regulate Fps1 phosphorylation is not completely rejected. In fact, it appears that Hog1 phosphorylates T231 within the N-terminus of Fps1. Hog1 is then recruited to phosphorylate Rgc2, which interacts with the C-terminus of Fps1, and thereby evicts Rgc2 and closes the channel. We believe that Slt2 phosphorylates S537 within C-terminal regulatory domain of Fps1. Perhaps this phosphorylation strengthens the Rgc2-Fps1 binding, hence supports the opening of Fps1 channel. Future investigation should focus on generating more data on phosphorylation pattern of Fps1 and also its interaction with Slt2, which can improve the predictive

potential of the model. Furthermore, our model suggests that there is no mutual crosstalk between HOG and CWI pathways on the protein kinase level. Rather it appeared that the two pathways stimulate each other by the response that they activate, i.e. each pathway generates the osmotic stimulus for the other pathway.

The adaptation of the yeast *S. cerevisiae* to hyperosmotic stress has been studied extensively but exclusively in medium with glucose as a carbon source. However, since yeast may have to cope with osmotic stress in absence of glucose, we investigated osmoregulation in presence of ethanol as a carbon and energy source. The results showed that osmoregulation in ethanol-grown cells are very similar that to glucose- grown cells with the exception of the compatible osmolyte. Ethanol-grown cells do not accumulate glycerol under hyperosmotic stress but accumulate trehalose instead. Although glycerol production requires less energy than that of trehalose, ethanol-grown cells ignore the option to accumulate glycerol. Understanding the reasons for this choice requires further studies. It also will be interesting to test if ethanol-grown cells use glycerol as osmolyte when it is externally supplied and how the Fps1 glycerol channel is regulated in this condition. It also appeared that gene expression profiles are less dependent on Hog1. Therefore, studying global gene expression profile may help determining the role of other metabolic pathways in osmoadaptation of ethanol-grown cells.

Taken together, in this thesis I investigated the quantitative characteristics of osmoregulation in the yeast *S. cerevisiae*. Applying different technologies for quantitative measurements in single cells and at population level, we provided time-resolved data of several aspects of osmoregulation, such as cell volume loss and recovery, sub-cellular protein shuttling, protein diffusion rate, protein phosphorylation, glycerol accumulation, and gene expression in order to better understand the interdependence and timeline of different events of osmo-adaptive responses.

10 Summary of articles

In this thesis, experimental observations are discussed in their appropriate context. However, below is a short summary of the results from the different papers.

Paper I:

We showed that Hog1 signaling (including Hog1 phosphorylation and nuclear localization) is delayed under severe hyperosmotic stress. Also nuclear translocation of the stress-responsive Msn2 transcription factor is delayed in severe hyper-osmotic stress, which suggests that the observed delay is a general phenomenon in response to higher stress levels. To investigate the mechanism of the signaling delay, we monitored cell volume loss and recovery in response to different degrees of osmo-shock in wild type, *gpd1*Δ *gpd2*Δ, *ptp2*Δ, *ptp3*Δ, and *fps1*Δ mutants. We observed that the timing of Hog1 nuclear accumulation correlates with cell volume recovery dynamics. The correlation between Hog1 nuclear translocation rate and the volume changes demonstrates that cellular compression slows down Hog1 signaling. Furthermore, we measured Hog1 diffusion rates and the rate of Hog1 nuclear transport under mild and severe hyper-osmotic stress and found that the diffusion rate of Hog1 in the cytoplasm is dramatically reduced in cells under strong osmotic stress. However, the Hog1 nuclear import rate is similar for cells exposed to different osmotic stress conditions. Taken together, we suggested that cell volume compression causes molecular crowding, which reduces signal progress. On the other hand, Hog1 nuclear translocation is more delayed compared to Hog1 phosphorylation and Msn2 nuclear accumulation. We proposed that under high stress levels, cytosolic targets may be prioritized in the early stages of adaptation.

Paper II:

We rewired Hog1-dependent osmotic stress-induced gene expression under the control of Fus3/Kss1 MAPKs in *hog1Δ* cells and showed that osmotic up-regulation of *GPD1* and *GPP2*, compounds involved in glycerol biosynthesis, is sufficient for synthetic osmoadaptation. Furthermore, upregulation of an unphosphorylatable, hyperactive form of *GPD1* (*GPD1*^{4A}), leads to an increase in accumulation of glycerol as well as better cell volume recovery as compared to *GPD1*. We also revealed that presence or absence of Fps1 in the synthetic osmoadaptation strain (*hog1Δ* with PFUS1-*GPD14A* PFUS1-*GPP2*)

has no effect on osmosensitivity of cells. However, introduction of unregulated *FPS1-Δ1* into the same strain caused a strong osmosensitive phenotype. This result suggests that although Fps1 gating is required for osmoadaptation, Hog1 is dispensable for Fps1 regulation under hyperosmotic stress. Therefore, we proposed that other kinases may be involved in regulation of Fps1 gating. Moreover, we showed that abnormal morphology of *hog1Δ* caused by hyperosmotic stress does not affect osmoadaptation.

Paper III:

We investigated Hog1 and Slt2 activation in response to hyperosmotic exposure followed by hypo osmotic stress. We combined mathematical modeling with experimental investigation to understand the regulatory mechanism of osmo-homeostasis in yeast. Our results showed that in contrast to Hog1 activation, the volume activation threshold for Slt2 is not fixed. Instead, the Slt2 activation threshold is regulated by a sensitizing mechanism. Furthermore, we studied potential crosstalk between HOG and CWI pathways at the protein kinase level or through Fps1 glycerol channel. Our results do not support a HOG and CWI crosstalk on protein kinase level. Instead, we suggested that these two pathways stimulate each other by the response they activate. On the other hand, we showed that in our experimental set up, deactivation of Hog1 caused by a shift to hypo osmotic condition might be sufficient for Fps1 regulation. We suggested that the role of Hog1 on Fps1 regulation is much more important than that of Slt2. Therefore we conclude that the HOG pathway regulates the main mechanisms of osmoadaptation in both hyper and hypo osmotic stress.

Paper IV:

We showed that upon arsenite exposure, the Slt2 MAPK is phosphorylated through Bck1. In addition, we demonstrated that Slt2 activation and its kinase activity are required for arsenite tolerance. Furthermore, we revealed that *Fps1* overexpression improves growth of wild type cells and also *wsc1*Δ, *mid2*Δ, and *rlm1*Δ mutants upon arsenite exposure, while it has no effect on arsenite sensitivity of *slt2*Δ and *bck1*Δ mutants. On the other hand, we demonstrated that simultaneous overexpression of *SLT2* and *FPS1* complements the arsenite-sensitive phenotype of the *slt2*Δ mutant, but not that of the *bck1*Δ mutant. These data show that Slt2 plays an essential role in arsenite detoxification upon *FPS1* overexpression. We further demonstrated that Slt2 affects the transport activity of Fps1 and mediates arsenite tolerance.

Paper V:

We investigated the response of yeast to hyperosmotic stress when cells were grown in a medium with ethanol as carbon source. Activation of the HOG pathway is required for osmotic adaptation in ethanol-grown cells and the Hog1 phosphorylation profile is similar in both glucose and ethanol medium. However, it seems that the total level of Hog1 is lower in ethanol medium. In addition, we revealed that unlike in glucose, ethanol-grown cells do not accumulate glycerol in response to hyperosmotic stress. Instead it appears that trehalose accumulation is increased in these cells. Volume recovery of cells growing in ethanol medium is much slower as compared to glucose-grown cells. The expression of osmoregulated genes in ethanol-grown cells seem to have the same profile as ethanol-grown cells, although it is less dependent on Hog1.

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