UNIVERSITY OF GOTHENBURG

Stress, Homeostasis and Robustness: Molecular and Systems Level Analysis in Yeast

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Abstract

Cells constantly encounter stress due to alterations in the external milieu or internal parameters. However, cells are robust to such changes and maintain homeostasis. Using the yeast *Saccharomyces cerevisiae* as a model organism, we attempted to elucidate aspects of homeostasis and robustness at both molecular and systems levels. At the molecular level, we focused on the aquaglyceroporin Fps1 and at the systems level we investigated the High Osmolarity Glycerol (HOG) pathway. Although Fps1 plays a key role in osmotic regulation and homeostasis, the mechanisms controlling Fps1 are not yet fully understood. We present evidence that Hog1 restricts the flux of glycerol and arsenite through Fps1 by phosphorylation of a residue within the N-terminal regulatory domain. This is the first report of a Mitogen Activated Protein Kinase (MAPK) regulating an aquaglyceroporin. In addition, we demonstrate that yet another MAPK, Slt2 may also be involved in regulating Fps1. It appears that Slt2 controls Fps1 by stimulating Fps1-mediated efflux. A residue within the C-terminal regulatory domain seems to be involved in controlling arsenite efflux through Fps1. Moreover, we show that in addition to the N- and C- termini, the transmembrane core of the protein also has a large effect on the transport activity of Fps1. Taken together, we speculate that phosphorylation of the termini affects the orientation of the transmembrane helices, thereby disturbing the transport activity of the protein. At the systems level we challenged the yeast HOG signal transduction pathway with systematic perturbation in the expression levels of its components under various external conditions to identify fragile nodes. We observed a high frequency of fragile nodes within the HOG pathway due to the inherent nature of signal transduction and the distribution of these fragile nodes was independent of function or location in the pathway topology. Moreover, the fragility patterns were mainly independent of the overall pathway activation status in response to different stresses. We found that the toxicity upon overexpression is at least partly due to pathway hyperactivation and can be suppressed by deletion of upstream or downstream pathway components. Furthermore, *in silico* analysis highlighted the impact of model structure on *in silico* robustness, and suggested complex formation and scaffolding as important contributors to the observed fragility patterns. Collectively, we believe that the robustness analysis can provide complementary information to dynamic data improving understanding of the operation of cellular signalling networks.

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The thesis is based on the following papers:

I. M. Thorsen, Y. Di, C. Tängemo, M. Morillas, D. Ahmadpour, C. Van der Does, A. Wagner, E. Johansson, J. Boman, F. Posas, R. Wysocki, and M. J. Tamás- The MAPK Hog1p Modulates Fps1p-dependent Arsenite Uptake and Tolerance in Yeast. Molecular Biology of the Cell, Vol. 17, 4400–4410, 2006

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

III. C. Geijer*, D. Ahmadpour*, M. Palmgren, C. Filipsson, M. J. Tamás, S. Hohmann, K. Lindkvist-Petersson- Yeast aquaglyceroporins use the transmembrane core to restrict glycerol transport. *Submitted manuscript for JBC*
* Equal contribution

IV. M. Krantz, D. Ahmadpour*, L.G. Ottosson*, J. Warringer, C. Waltermann, B. Nordlander, E. Klipp, A. Blomberg, S. Hohmann, and H. Kitano- Robustness and fragility in the yeast high osmolarity glycerol (HOG) signal-transduction pathway. Molecular Systems Biology 5; Article number 281, 2009
* Equal contribution

Studies, which are not included in this thesis:

V. C. Walterman, D. Ahmadpour, et al. - Characterisation of the robustness landscape of the core-signalling network of baker's yeast. *Manuscript in preparation*

VI. M. Popovic, IS. Møller, P. Pedas, D. Ahmadpour, M. Yacout, MJ. Tamás, TP. Jahn and JK Schjørring- AtTIP4;1 is bi-directional arsenite channel involved in arsenite accumulation in *Arabidopsis*. *Manuscript in preparation*

Contents

1. Introduction

My research aimed to provide a better understanding of the mechanisms, with which cells cope with stress at both molecular and systems levels. Cells are constantly facing and responding to changes in the environment, such as fluctuations of the chemical and physical properties of the extracellular milieu and changes in intracellular parameters. Signal transduction pathways are central to a cell's ability to respond and adapt. Signalling pathways are activated by specific stimuli and distinct regulatory signals are produced in the form of activated signalling pathway components. Despite the different types of fluctuations encountered by cells, they maintain homeostasis, which seems to be an intrinsic feature of life and vital for viability and fitness. Altered osmolarity is a very common and frequently occurring stress factor, in spite of which cells remain osmohomeostatic. I have focused on the regulation of the transmembrane aquaglyceroporin Fps1, which plays a critical role in osmohomeostasis by controlling the glycerol balance in the cell. Apart from its important role in osmo-regulation, Fps1 is also known to be a major entry route into yeast cells for the trivalent metalloid arsenite. Arsenic is a carcinogenic and teratogenic element that is responsible for severe water contamination problems in some parts of the world. However, it is also used to treat cancer and parasitic infections. Thus, elucidation of the mechanisms behind Fps1 regulation is imperative for understanding osmo- regulation and homeostasis as well as for therapeutic and decontamination applications. Apart from studying homeostasis and robustness at molecular level, we also studied robustness at systems level. As a model, we first focused on the extensively studied HOG pathway and later expanded the study to additional signalling pathways in yeast, applying a genetic screening approach based on overexpression of components.

In Paper I, we describe a novel role for Hog1 in protecting cells during exposure to arsenite. We show that Fps1 is phosphorylated *in vivo* on threonine 231 within the N-terminal domain and this phosphorylation critically affects Fps1 activity. The results suggest that Hog1 possibly affects Fps1 phosphorylation at T231 and this phosphorylation negatively modulates the activity of Fps1 under basal condition and upon exposure to arsenite.

In Paper II, we show that Slt2 is phosphorylated in response to arsenite. We propose that Slt2 contributes to arsenite tolerance, possibly by stimulating arsenite efflux through Fps1. Our data further suggest residue S537 within the C-terminal tail of Fps1 as a candidate target for Slt2.

In the work described in Paper III, we made use of the regulated *Saccharomyces cerevisiae* Fps1 and hyperactive *Ashbya gossyppii* Fps1. By domain swapping between these two Fps1 orthologs and employing an intragenic suppressor screen, we obtained evidence that, in

addition to the N- and C- terminal tails, the transmembrane core of the protein plays a role in the gating of Fps1.

In Paper IV, we discuss the effect of systematically perturbing the HOG pathway by increasing expression levels of its components under various external conditions and subsequently monitor robustness. To this end, we have applied the genetic Tug of War (gToW) overexpression approach. We observed that expression changes have a very strong impact on the HOG pathway. Fragile nodes had diverse biochemical functions, were scattered throughout the pathway topology and were largely independent of pathway activation by external stimuli. Toxicity at least partly was triggered by pathway hyper activation, as the strongest phenotypes caused by Pbs2 and Ssk1 were suppressed by the deletion of *HOG1*. We also demonstrate that the structure of the mathematical model highly affects the apparent fragility of different nodes since *in silico* analysis suggested that complex formation and scaffolding are important contributors to the observed fragility pattern.

In a fifth study (not included in this thesis), we aimed to investigate network robustness against changes in expression levels and map interconnections of signalling pathways. Using gToW method, we challenged five conserved signal transduction pathways, the cAMP-protein kinase A (PKA), the protein kinase C (PKC), the target of rapamycine (TOR), the pseudohyphal differentiation (PHD) and the mating (MAT) pathways to identify nodes of fragility and robustness. Thus far, we have observed that about 70% of the nodes are fragile with effects ranging from moderate but significant to virtually fatal. These effects spread over all five pathways and encompass both positive and negative regulators of the pathways. We also determined the levels of interconnectivity among the signal transduction pathways by tracing the toxicity effect of fragile nodes with deletion mutants of the key components impairing each network module. We observed a substantial albeit incomplete interpathway suppression. We are currently developing a theoretical framework to visualise, interpret and disseminate the results.

In a sixth study (also not included in the thesis) we addressed the serious problem of arsenic accumulation in plants. We investigated if the tonoplast intrinsic protein 4;1 (TIP4;1) from *Arabidopsis thaliana* has any role in arsenite uptake. Growth and arsenite uptake assays following heterologous expression of AtTIP4;1 in yeast showed that AtTIP4;1 mediates arsenite influx into yeast.

The background information, past and novel results will be discussed in detail in subsequent parts of the thesis, which will hopefully provide a framework for forthcoming studies.

2. Stress, homeostasis and robustness

Robustness and homeostasis are fundamental features of biological systems. Robustness refers to the preservation of the functionality of a system to a certain degree despite external or internal changes (Kitano 2004; Stelling et al. 2004; Kitano 2007). Moreover, the biological system is homeostatic if the state of the system remains stable. These two aspects of biological systems are inter-linked if maintaining the state of the system allows its function to be maintained (Kitano 2011). Yeast cells as single celled organisms are homeostatic, robust biological systems, which live freely in nature, thereby experiencing extreme environmental variations such as fluctuating salinity, desiccation, freezing, changes in pH as well as changes in internal parameters such as nutritional and energy status or stochastic fluctuations in gene expression levels. If any of the extrinsic or intrinsic conditions hamper cellular homeostasis and robustness, is referred to as cell stress. To cope with these stresses, rapid acting and efficient adaptation mechanisms are vital to maintain viability and maximal proliferation capacity. One way to achieve robustness and homeostasis is to adapt to external changes by triggering a series of cellular responses. The sensing and responding mechanism typically begins at the plasma membrane, where sensors sense stimuli, generate an internal signal and trigger the intracellular response. Signal transduction pathways produce distinct regulatory signals that eventually lead to adaptation of cell growth and proliferation as well as adjustment of the gene expression program and other features of cell (Hohmann and Mager 2003).

3. Water- and osmo- homeostasis

The ability to adapt to sudden changes in water activity is a fundamental property of living cells. Water activity is defined as the chemical potential of free water in solution and is a measure of how efficiently water can partake in a chemical reaction (Hohmann 2002). Changes in the concentration of dissolved molecules in the medium, referred to as osmotic stress, lead to alterations in water activity, thereby shifting the equilibrium of the biochemical reactions inside the cell (Hohmann 2002). Upon an increase in medium osmolarity (decreased water activity), water flows out of the cell towards higher solute concentration. This results in a decrease in cell volume, and an increase in the concentrations of all the substances present in the cytoplasm. Likewise, a decrease in external osmolarity (hypoosmotic stress) leads to water inflow into the cell and consequently an increase in cell volume and a decrease in concentration of solutes (Csonka and Hanson 1991; Blomberg and

Adler 1992; Hohmann and Mager 2003). If cells could not adapt to changes in osmolarity, this would eventually result in an arrest in cellular activities.

In order to maintain an appropriate cell volume and free to bound water ratio for biochemical reactions, cells have a higher internal osmotic pressure compared to the surrounding medium. This pressure is counteracted by cell wall resistance and referred to as cell turgor. Upon a change in external osmolarity, cells must adapt the internal osmolarity to maintain the turgor pressure (Wood 1999; Hohmann 2002; Hohmann and Mager 2003). The accumulation of osmolytes is a conserved strategy among all kingdoms of life. Osmolytes to some extent replace water, protect biomolecules, and increase the intracellular water potential thereby drawing water into the cell (Hohmann 2009). The nature of the osmolyte varies among different organisms ranging from ions like potassium, amino acids and derivatives, polyols and sugars, methylamines and methylsulphonium compounds to urea (Yancey et al. 1982; Yancey 2005). Some organisms use only one osmolyte, whereas others use several. In proliferating *S. cerevisiae* cells, glycerol serves as the compatible solute in order to maintain shape, turgor as well as appropriate water and ion concentration for optimal functioning (Brown 1978; Blomberg and Adler 1992; Hohmann 2009). Glycerol, a three-carbon polyhydroxy alcohol, can be taken up and utilised as a carbon and energy source. It is also a major by-product of sugar fermentation to ethanol in *S. cerevisiae*. Under anaerobic conditions, glycerol synthesis is essential for maintaining the cellular redox balance as synthesis of glycerol requires NADH and regenerates NAD^+ (Yancey 2005) . In addition to its crucial role in osmoadaptation, glycerol protects against high temperature (Siderius et al. 2000) and oxidative stress (Påhlman et al. 2001).

Several signalling pathways such as HOG and Cell Wall Integrity (CWI) pathways, allow the cell to perceive and respond quickly and precisely to osmolarity alterations in yeast (Hohmann and Mager 2003)(will be discussed in further details below). Accumulation of glycerol is regulated by Hog1, the Mitogen Activated Protein (MAP) kinase of the HOG pathway, via several different mechanisms. Hog1 regulates the expression of Stl1, a glycerol/H+ symporter (Rep et al. 2000). Expression of the genes encoding Gpd1 (NAD-dependent glycerol-3-phosphate dehydrogenase), Gpp1 (constitutively expressed isoform of DL-glycerol-3-phosphatase; involved in glycerol biosynthesis) and Gpp2 (DL-glycerol-3-phosphatases involved in glycerol biosynthesis) is up regulated in a partly Hog1-dependent manner upon hyper osmotic shock (Rep et al. 2000). Hog1 is also involved in short-term regulation of glycolysis by increasing the rate of glycerol production via effects on the activity of the enzyme 6-phosphofructo-2-kinase encoded by *PFK2* (Dihazi et al. 2004). This enzyme produces fructose-2,6-bisphosphate, an activator of the glycolytic phosphofructokinase. Together with glycerol synthesis, the intracellular glycerol content is regulated via the glycerol channel Fps1, which undoubtedly plays an essential role in controlling glycerol transport. Mutations that render Fps1 unregulated, lead to glycerol leakage and a diminished ability to

adapt to hyperosmotic stress. Upon hyperosmotic shock, Fps1 channel inactivation/ closure ensures glycerol accumulation, whereas a drop in osmolarity (hypoosmotic shock) leads to re-activation/opening of Fps1 to release glycerol and maintain proper turgor pressure and osmohomeostasis (Figure 1) (Tamas et al. 1999). In this thesis, novel regulatory mechanisms that control Fps1 activity will be discussed later.

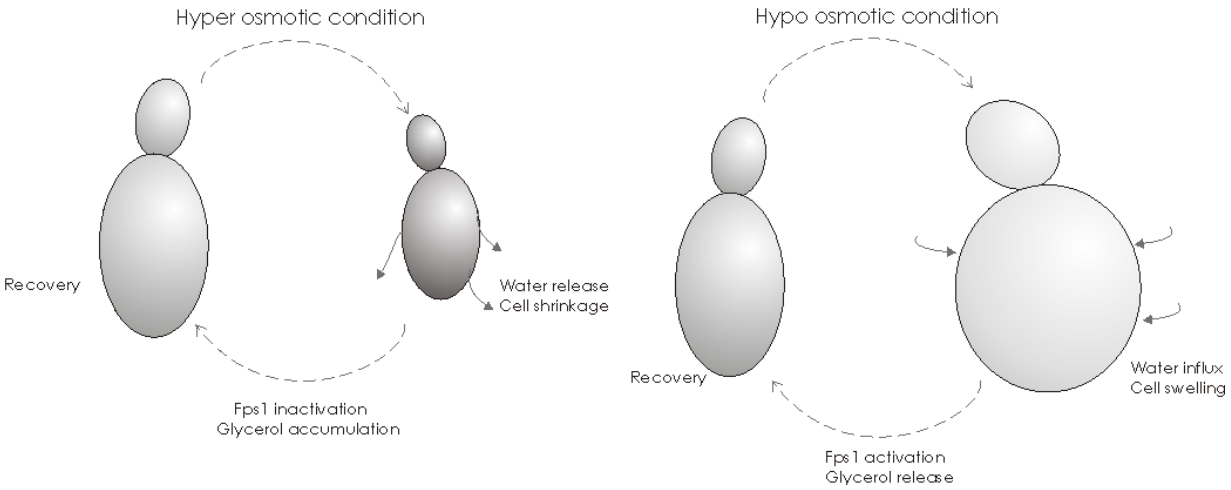


Figure 1. Representation of osmohomeostasis in *Saccharomyces cerevisiae*
Upon hyperosmotic stress, water flows out of the cell towards the higher solute concentration, which results in a decrease in cell volume. Fps1 channel inactivation/ closure results in glycerol accumulation, thereby proper turgor pressure and volume recovery. A decrease in external osmolarity (hypoosmotic stress) leads to water inflow into the cell and as a consequence an increase in cell volume. Re-activation/opening of Fps1 ensures release of glycerol and proper turgor pressure.

4. Signal transduction and MAPK pathways in yeast

Cells use signal transduction pathways to sense and respond to changing environmental conditions and extrinsic or intrinsic stimuli. The Mitogen Activated Protein Kinase (MAPK) cascade is a commonly employed cellular signalling module. A canonical MAPK module includes three sequential kinases, comprising a MAPK Kinase Kinase (MAPKKK), MAPK Kinase (MAPKK) and MAPK (Widmann et al. 1999). The MAPKKKs contain a C-terminal serine/threonine protein kinase domain, which upon activation phosphorylates two serine or threonine residues of its MAPKK target. MAPKK is a dual specificity serine/ threonine and tyrosine protein kinase that phosphorylates the threonine and tyrosine residues of a conserved Thr- X- Tyr motif in the activation loop of its target MAPK, which then upon phosphorylation is often transferred from cytosol to nucleus. MAPKs in turn phosphorylate a diverse set of substrates, including transcriptional factors, translational regulators, enzymes

and other classes of the proteins. In this manner, MAPKs control gene expression, metabolism, cell cycle progression, cell growth and morphogenesis (Widmann et al. 1999; Schwartz and Madhani 2004; Chen and Thorner 2007). Inappropriate activation of MAPK pathways commonly causes deleterious effects, and hence signalling is strictly regulated (Martin et al. 2005). Since activation of MAPKs requires phosphorylation, one effective mechanism of negative regulation/inactivation is dephosphorylation by protein phosphatases of the serine/threonine, tyrosine, or dual- specificity classes (Martin et al. 2005; Chen and Thorner 2007).

Thus far, it is known that *S. cerevisiae* encodes four MAPKKKs (Ste11, Bck1, Ssk2 and Ssk22), four MAPKKs (Ste7, Mkk1, Mkk2 and Pbs2), six MAPKs (Fus3, Kss1, Slt2/Mpk1, Smk1, Hog1 and YKL161C), among which only Fus3, Kss1, Hog1 and Slt2 are considered true MAPKs. Two protein tyrosine phosphatases (Ptp2 and Ptp3), three serine/threonine phosphatases (Ptc1, Ptc2 and Ptc3) and two dual-specificity phosphatases (Msg5 and Sdp1) control the MAPKs in yeast (Martin et al. 2005; Chen and Thorner 2007). This means that cells respond to a wide variety of stimuli using a relatively limited set of signal transduction elements. Many of the signalling components are shared between different pathways, which could potentially lead to cross-activation, where an improper downstream pathway is activated following an external stimulus. Nevertheless, stimulus-output fidelity is firmly maintained (Schwartz and Madhani 2004; McClean et al. 2007). Specificity is achieved via different mechanisms, which can be grouped into four main categories: 1- sequestration (physical insulation of shared components), 2-combinatorial signalling (coincidence of two different inputs necessary for pathway activation), 3-cross pathway inhibition (Schwartz and Madhani 2004) and 4-kinetics (duration, magnitude of the activation and accurate intensity of signalling, which are partly modulated by controlling phosphorylation status of MAPKs) (Martin et al. 2005).

S. cerevisiae contains four known MAPK pathways. The MAPK pathway, by which *S. cerevisiae* orchestrates the response to pheromone is called the mating pheromone response pathway and involves the MAPK Fus3. Activation of Fus3 leads to expression of numerous mating-specific genes, imposing cell-cycle arrest, promoting polarised cell growth, establishing changes in the plasma membrane and cell wall necessary for cell–cell fusion, and orienting the nucleus and modifying its envelope to permit fusion of the two haploid nuclei (Chen and Thorner 2007). In addition to Fus3, the MAPK Kss1 is also transiently activated upon pheromone stimulation. Double mutation of *FUS3* and *KSS1* leads to cell sterility, whereas the presence of either of them permits mating. This phenotype suggests that Fus3 and Kss1 have partially redundant functions, but Fus3 plays the major role in pheromone response (Chen and Thorner 2007). The qualitatively different contributions of Fus3 and Kss1 to mating might be explained by the differences in substrate preference or the temporal and spatial dynamics of the two MAPKs (Chen and Thorner 2007).

Upon nitrogen starvation, diploid *S. cerevisiae* undergoes morphological changes and shifts to filamentous or pseudohyphal growth. The shift is accompanied by cell elongation, unipolar budding, attachment of daughter to mother cells and the ability to invade semisolid media. Haploid cells exhibit invasive growth, which is stimulated by glucose starvation. The shift to pseudohyphal growth seems to be controlled by several parallel signaling pathways, one of which involves a MAPK cascade that shares many components with the mating pheromone pathway and the MAPK Kss1 (Widmann et al. 1999; Schwartz and Madhani 2004). In addition, the protein kinase A (PKA) pathway plays a crucial role in pseudohyphal and invasive growth (Chen and Thorner 2007).

Upon starvation of both carbon and an additional essential nutrient, such as nitrogen, diploid yeast cells enter meiosis and generate four haploid spores. Spores are specialised cells coated with a spore-wall composed of four layers. The spore-wall together with intracellular adaptations enables the spore to withstand harsh environmental conditions. The MAPK Smk1 is required for spore-wall formation. Diploid yeast cells lacking the MAPK Smk1 undergo meiosis but exhibit defective assembly of the outer two spore-wall layers (Huang et al. 2005; Chen and Thorner 2007). Upstream MAPKKK and MAPKK for Smk1 have not been described.

The MAPKs Hog1 and Slt2 are discussed in more detail in the following sections.

4.1. High Osmolarity Glycerol (HOG) pathway (Paper I and Paper IV)

The yeast HOG pathway mediates adaptation of yeast cells to increased external osmolarity. Its most important role in osmotic adaptation concerns the control of glycerol accumulation (Hohmann 2002; Hohmann 2009). The pathway consists of two branches, the Sln1 and the Sho1 branch, which converge on the MAPKK Pbs2. The Sln1 branch involves a histidine-aspartate phospho-relay module similar to two-component signalling systems in bacteria (Posas et al. 1996). The module consists of three components: Sln1, Ypd1 and Ssk1. Sln1 is a plasma membrane localised sensor. It is active under ambient conditions and inactive upon hyperosmotic shock. Sln1 has an extracellular sensor domain, a cytoplasmic histidine kinase domain and a receiver domain (Posas and Saito 1998). Under iso-osmotic conditions, when Sln1 is active, this phospho-relay system initiates by autophosphorylation of Sln1 on His576. This phosphate is then transferred to Asp1144 on Sln1, and subsequently to His64 on the transfer protein Ypd1. Ypd1 in turn transfers the phosphate group to Asp554 on the response regulator Ssk1 (Posas et al. 1996). Ssk1 phosphorylation prevents interaction of Ssk1 with the MAPKKKs Ssk2 and Ssk22 (Posas and Saito 1998). Under hyperosmotic conditions, when the Sln1-Ypd1-Ssk1 module is inactive, unphosphorylated Ssk1 binds to the regulatory domain of Ssk2 and Ssk22, allowing them to autophosphorylate and activate

themselves (Saito and Tatebayashi 2004). Two mucine-like transmembrane sensors, Msb2 and Hkr1, are the osmosensors of the Sho1 branch (Tatebayashi et al. 2007). Following sensor stimulation by hyperosmotic stress, Pbs2, which acts as a MAPKK in the pathway but also serves as a scaffold protein in the Sho1 branch, is recruited to the plasma membrane. Pbs2 carries along Ste11, which thereby co localises with the Ste20 and Cla4 kinases, located at the plasma membrane in association with the Cdc42 G-protein. Phosphorylation of Ste11 by either Ste20 or Cla4 activates Ste11 (Tatebayashi et al. 2007; Hohmann 2009). Once active, Ssk2, Ssk22 and Ste11 phosphorylate the MAPKK Pbs2. Pbs2 in turn phosphorylates and activates the MAP kinase of the pathway, Hog1 (Figure 2). Either branch in isolation can activate Hog1 upon hyperosmotic stress. The Sln1 branch has a more prominent role and appears to be activated by relatively small osmotic changes, while the Sho1 branch appears to have a role in coordinating signalling between HOG and other MAPK pathways (Elion et al. 2005; Hohmann 2009). Phosphorylation of Hog1 is required for Hog1 import into the nucleus, where the transcription factors Hot1, Sko1, Msn2/4, Msn1 and Smp1 seem to be involved in Hog1-dependent responses (Hohmann et al. 2007). Hot1 controls the genes encoding glycerol metabolism GPD1/GPP2 and glycerol uptake STL1. Msn2 and Msn4 are redundant transcription factors involved in general stress responses, and Sko1 controls the expression of several regulators of the osmostress response, such as the protein tyrosine phosphatase Ptp3 and transcription factor Msn2 (Hohmann et al. 2007). Interestingly, by tethering Hog1 to the plasma membrane to prevent Hog1 nuclear localisation, it has been demonstrated that activation of gene expression is not critical for osmoadaptation since the cells can survive long-term hyperosmotic challenges of ionic and non-ionic solutes without exhibiting the normal change in transcriptional programme (Westfall et al. 2008). Hog1 also has numerous cytoplasmic targets, e.g. Nha1 Na+/H+ antiporter, Tok1 potassium channel (Proft and Struhl 2004), protein kinases Rck1 and Rck2 involved in osmotic and oxidative stress responses, and inhibitor of cyclin-dependent protein kinase Sic1, suggesting that a fraction of Hog1 remains in the cytosol even though nuclear accumulation is apparent (Elion et al. 2005; Bicknell et al. 2010).

Following a hyperosmotic shock, Hog1 activity needs to be down regulated as constitutive active Hog1 is lethal (Maeda et al. 1993). In a robustness study on HOG pathway, we showed that overexpression of some components of the HOG pathway, which resulted in lethality, was in fact due to hyperactivation of Hog1 and could be suppressed by deletion of downstream components (Paper IV, discussed in respective section). The lethality associated with Hog1 hyperactivity appears to be due to an inhibitory effect on cell- cycle progression (Escote et al. 2004; Adrover et al. 2011). The phosphorylation state of Hog1 is controlled by a number of different phosphatases, including the protein tyrosine phosphatases Ptp2 and Ptp3 (Mattison and Ota 2000) and protein serine phosphatase Ptc1 (Warmka et al. 2001) and possibly Ptc2 and Ptc3 (Hohmann 2009). Although dephosphorylation of Hog1 by

phosphatases plays an important role in down regulation of Hog1 activity, mathematical modelling and simulation of the feedback control system suggested that this alone cannot explain the adaptation. Instead, it appears that osmotic adaptation determines the period of HOG pathway activation. This theory is supported by several experimental observations. For instance, cells overexpressing *GPD1* with an increased intrinsic glycerol production capacity, show more transient HOG pathway activation (Krantz et al. 2004). Moreover, it has been shown that the pathway becomes inactivated within about 5 minutes under conditions of low osmotic stress, when a small amount of glycerol production is required for osmotic adaptation. In contrast, severe osmotic stress, which requires a larger increase in the internal glycerol concentration, shows more prolonged pathway activity (Pelet et al. 2011). Further evidence has been provided by studies of mutants with decreased ability to accumulate glycerol, such as a truncated version of Fps1, which exhibits a relatively long period of Hog1 activity (Siderius et al. 2000). Importantly, Hog1 also displays basal signalling, which seems to depend on the Sln1 branch and is under constant negative regulation, which itself is dependent on Hog1 kinase activity (Macia et al. 2009). Phosphatases are good candidates for this regulation as the *ptc1⊿ptp2⊿* double mutant shows lethality due to continuous Hog1 activity (Maeda et al. 1993). Interestingly, in our robustness study, we observed that lethality caused by overexpression of *PBS2* and *SSk1* could even be suppressed by deletion of *SSK2,* which is an upstream component (Paper IV, Suppl. Figure 6D). This again suggests that there is a constitutive basal signal that originates upstream of Ssk2, possibly Ssk1, which is amplified upon *PBS2* or *SSk1* overexpression and leads to lethality (Paper IV).

Apart from the importance of HOG basal signalling in providing higher efficiency, faster response and higher sensitivity to variations in external signals (Macia et al. 2009), we have further shown (Paper I) that this basal signalling plays an important physiological role in osmohomeostasis. In the condition that Fps1 has been shown to be the main route for glycerol uptake, we compared the glycerol influx in wild-type (WT) and a *hog1⊿* mutant. Interestingly, *HOG1* deletion resulted in a 2–3-fold increase in glycerol uptake compared to WT indicating that Hog1 basal signaling restricts Fps1 transport activity under normal conditions (Paper I).

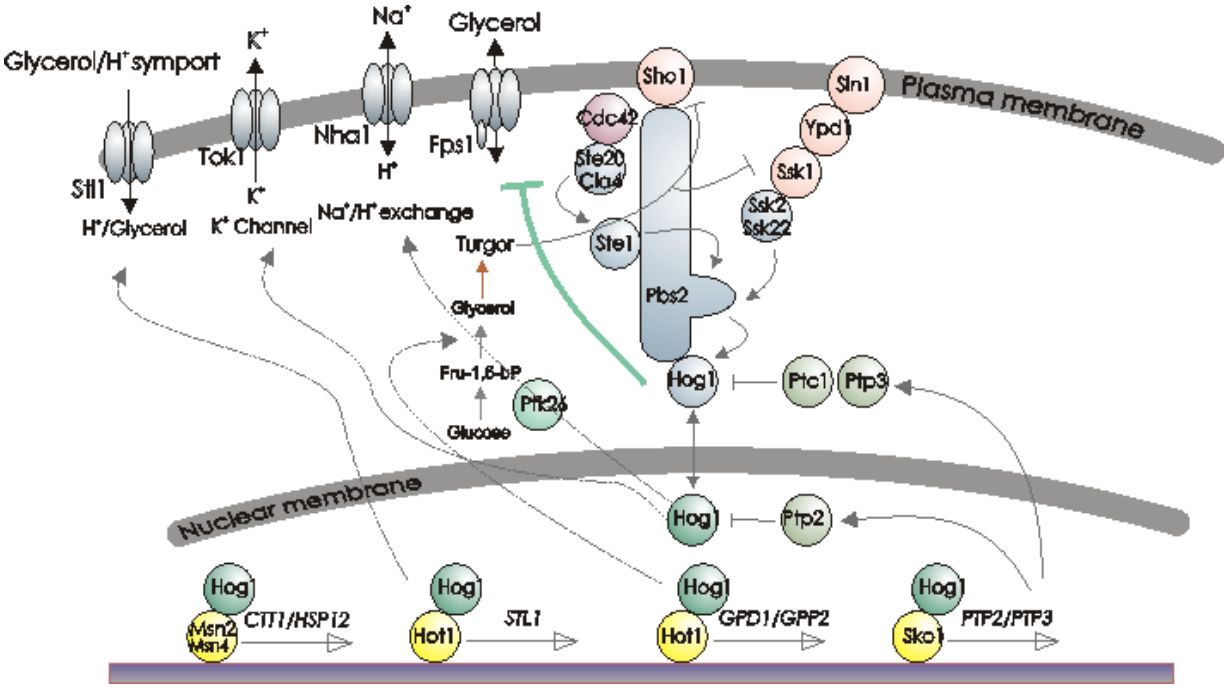


Figure 2. Components, flow of information and overview of response mechanisms in the yeast HOG pathway

The HOG pathway consists of two branches: the Sho1 branch and the Sln1 branch, which converge at the MAPKK Pbs2. Upon activation, Pbs2 phosphorylates/activates Hog1. Hog1 has both cytoplasmic and nuclear targets. Nha1, Tok1, Stl1 and Fps1 are Hog1 cytoplasmic targets. Hog1 also appears to stimulate glycolytic flux by activating Pfk26. In the nucleus, Hog1 interacts with different transcription factors, such as Sko1, Hot1 and Msn2/4 to control different sets of genes. Ptc1, Ptp2/3 protein phosphatases negatively regulate Hog1. See text for further details. Adapted from the courtesy of Professor Stefan Hohmann, University of Gothenburg

It has been shown that Hog1 is also involved in responses to many other stimuli, although the nature of these responses differs widely. The HOG pathway is reported to be involved in responses to acetic acid (Mollapour and Piper 2007), cold (Hayashi and Maeda 2006; Panadero et al. 2006), heat (Winkler et al. 2002), citric acid (Lawrence et al. 2004), low external pH (Kapteyn et al. 2001) and oxidative stresses (Bilsland et al. 2004). We were the first to report that the HOG pathway contributes to arsenite tolerance and Hog1 is activated in response to exposure to arsenite (Paper I). Arsenite-mediated Hog1 phosphorylation displays quantitative and qualitative differences compared to phosphorylation during osmotic stress. While maximal Hog1 phosphorylation is observed after just 5 minutes and is no longer detectable after about 15 minutes in response to 0.4M NaCl, Hog1 phosphorylation in response to arsenite is weaker and lasts longer (Paper I, Figure 3). Moreover, we showed that activation/ phosphorylation of Hog1 does not lead to nuclear accumulation (Paper I, Figure

4), suggesting no significant Hog1 dependent transcriptional regulation, whereas Hog1 mediates arsenite tolerance through cytoplasmic target(s). Additional support for a role of Hog1 in arsenite tolerance was subsequently reported (Sotelo and Rodriguez-Gabriel 2006). The authors claimed that Hog1 is needed for tolerance to arsenite to facilitate transcriptional changes and that expression of *ACR3*, encoding the plasma membrane arsenite transporter, is stimulated in a Hog1- dependent manner in response to arsenite (Sotelo and Rodriguez-Gabriel 2006). However, our data indicate that Acr3 mediates arsenite efflux independently of Hog1 (Paper I, Figure 5). Inconsistency between these two findings might be because Sotelo and Rodriguez- Gabriel only examined one time point. *ACR3* induction is considerably slower at high arsenite concentration (Thorsen et al. 2007), and in a *hog1⊿* mutant, intracellular arsenite levels are higher than WT. Therefore, it is possible that induction of *ACR3* was delayed in the *hog1⊿* mutant and was observed as elevated at the particular time point they studied. We further demonstrated that arsenite- stimulated Hog1 phosphorylation is affected by deletion of upstream pathway components. In particular, the Sln1- Ssk1 branch appears to play a more prominent role (Paper I, Table 2). We also tested whether the kinase activity of Hog1 is critical for its contribution to metalloid tolerance. Our data indicate that neither a Hog1 variant that is unphosphorylatable by Pbs2, nor kinase-dead Hog1 can complement the sensitivity of *hog1⊿* in the presence of arsenite (Paper I, Figure 2A). Consistent with the data, we demonstrated that elevated Hog1 kinase activity in a mutant lacking the tyrosine phosphatases Ptp2 and Ptp3 has the opposite effect and leads to increased arsenite resistance (Paper I, Figure 2B). I will later discuss how Hog1 contributes to metalloid tolerance by affecting Fps1 transport activity.

4.2. Cell Wall Integrity (CWI) pathway (Paper II)

The CWI pathway is another signalling system, in addition to the HOG pathway, that responds to alterations in osmolarity in yeast (Hohmann and Mager 2003). In contrast to the HOG pathway, which is activated under hyperosmotic conditions, the CWI pathway is rapidly and transiently induced by a drop in osmolarity (Levin 2005; Levin 2011). Upon activation, the CWI pathway controls cell- surface remodeling (Hohmann 2009) and appears to aim to diminish turgor pressure on the cell wall (Hohmann 2002). The CWI pathway activation occurs through cell surface sensors Wsc1, Wsc2, Wsc3, Mid2 and Mtl1, of which Wsc1 and Mid2 play the most important role in sensing (Ketela et al. 1999; Levin 2011). Upon activation, plasma membrane phosphatidylinositol-4,5-*bis*phosphate($PI4,5P_2$) recruits Rom1/2 Guanine Exchange Factors (GEFs) to the plasma membrane, and then together with the sensors, stimulates nucleotide exchange on Rho1 (Levin 2005; Levin 2011). In its GTP bound form, Rho1 activates Pkc1. Activation of Pkc1 leads to activation of the downstream MAPK cascade, which consists of the MAPKKK Bck1 activated by Pkc1,

MAPKKs Mkk1/2 and MAPK Slt2 (Mpk1). Activated Slt2 in turn, activates two transcription factors Rlm1 and SBF complex. Rlm1 controls 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, which is a heterodimeric complex of two proteins, Swi4 and Swi6, is involved in the regulation of gene expression at the G_1/S transition (Levin 2011). Slt2 activity is downregulated by four protein phosphatases; Msg5, Ptp2, Ptp3 and Sdp1. Msg5 is a dual- specificity phosphatase and seems to undergo mutual regulation with Slt2. Slt2 phosphorylates Msg5 upon activation of the CWI pathway, reducing the affinity and association between Msg5 and Slt2, causing a positive feedback loop and prolonged activation of Slt2 (Flandez et al. 2004). The function of Msg5 with respect to the CWI pathway, is believed to be maintenance of a low basal activity of Slt2 in the absence of stress (Levin 2011). The Ptp2 and Ptp3 tyrosine phosphatases also dephosphorylate Slt2. However, Ptp2 appears to be more effective in dephosphorylating Slt2, where they seem to undergo a reciprocal regulation. Slt2 dependent induction of Ptp2 expression, provides a negative feedback loop for its inactivation by Ptp2. Stress-inducible dual-specificity MAPK phosphatase Sdp1 also negatively regulates Slt2 through direct dephosphorylation (Levin 2005; Levin 2011) (Figure 3).

Conventionally, the CWI pathway is known to be activated by conditions that confer cell-wall stress, damage or expansion. Therefore, it is implicated in polarised cell growth, heat and oxidative stress. It is also involved in response to treatment with glucanases e.g. Zymolyase, response to treatment with chitin binding agents e.g. Calcofluor white and response to pheromone treatment (Harrison et al. 2004; Levin 2005). Although these responses display different timings and kinetics (Hohmann 2002). In Paper II, we described a novel role for the CWI pathway contributing to arsenite tolerance. Cells deleted for *WSC1*, *BCK1* and *SLT2* were found to be sensitive to arsenite. Our findings contrast with a previous report claiming that Slt2 and Bck1 are not involved in the arsenite response (Sotelo and Rodriguez-Gabriel 2006). The discrepancy of these results might be explained by different arsenite stress severity (0.25mM compared with 1mM). We further showed that both activation of Slt2 by an upstream kinase and kinase activity of Slt2 are required for arsenite tolerance (Paper II, Figure 3). Slt2 is activated upon arsenite exposure, and this activation is Bck1 dependent. Slt2 phosphorylation appears to be induced by intracellular arsenite as Slt2 phosphorylation remains at basal levels in the *fps1*Δ mutant, in which the arsenite entry route is knocked out (Paper II, Figure 4). We further showed that Slt2 contributes to arsenite tolerance by positively affecting efflux through Fps1 (discussed below).

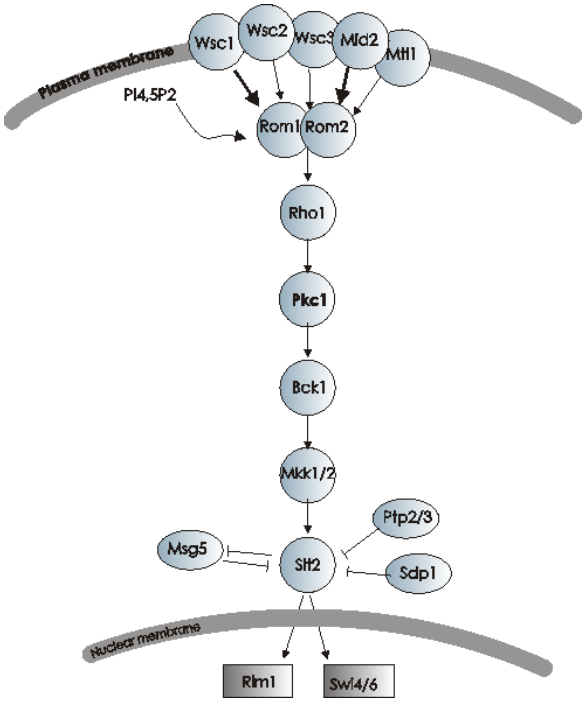


Figure 3. Cell wall integrity pathway

Signals are initiated at the plasma membrane via the cell surface sensors Wsc1/2/3, Mid2, and Mtl1. Together with PI4,5P_2 , which recruits the Rom1/2 GEFs to the plasma membrane, the sensors stimulate nucleotide exchange on Rho1. The relative input of each sensor is indicated by the width of the arrows. Rho1 activates the Pkc1 MAPK cascade, which comprises Bck1, Mkk1/2, and Slt2. Several MAPK phosphatases down regulate Slt2. Two transcription factors, Rlm1 and the SBF complex (Swi4/Swi6), are targets of the MAPK.

5. Aquaporin family

The aquaporin family, which facilitates the selective movement of water or other small molecules such as glycerol, has been shown to play a key role in osmoadaptation in yeast and many other organisms (Tamas et al. 1999; von Bulow et al. 2012). The phenomenon of cell-membrane water permeability had been debated for decades. It was initially believed that water passes thorough membrane lipid bilayers by simple diffusion. However, simple diffusion could not explain the high water permeability of some cells and tissues, such as red blood cells and renal tubules. The molecular mechanisms of water transport across cell membranes remained undefined until the discovery of the aquaporin family of water channels (Agre and Kozono 2003). The 28 kDa integral membrane protein now known as

AQP1 was first purified from red blood cells (Smith and Agre 1991). The ability of this protein to confer water permeability was tested in *Xenopus laevis* oocytes, which normally have a low water permeability (Preston et al. 1992). Studies on oocytes and reconstituted membranes have demonstrated finite water permeability in simple membranes and up to 100 times greater water permeability in AQP1 containing membranes. AQP1 is now widely known as the first recognised molecular water channel (Agre and Kozono 2003) and Peter Agre was awarded the Nobel Prize in Chemistry for the discovery of aquaporins in 2003.

Aquaporins are integral membrane proteins that belong to the larger Major Intrinsic Protein (MIP) family, which has been shown to contain functionally diverse members responsible for transporting water, glycerol, urea, arsenite, carbon dioxide, etc. (Hedfalk et al. 2006). Based on functionality, they are divided into two subcategories. Orthodox aquaporins mediate a rapid and highly selective flux of water across biological membranes, thereby playing an important role in the osmo-regulation of the cells and organisms. Aquaglyceroporins facilitate transmembrane transport of small neutral molecules, such as polyols, urea and arsenite besides water, thereby playing an important role in nutrient uptake, osmo-regulation and probably other processes (Hohmann et al. 2000; Hohmann S. et al. 2001; Tornroth-Horsefield et al. 2010). Aquaporins are found in Bacteria, Archea and Eukarya (Tanghe et al. 2006). They have an amazing variety of physiological functions including concentrating urine in kidney, maintaining eye- lens transparency, maintaining water homeostasis in the brain, facilitating a rapid response to osmotic shock in yeast (Hedfalk et al. 2006) and regulating water permeability and transport in response to variations in water availability in plants (Tanghe et al. 2006).

5.1. Aquaporin classification

5.1.1. Mammals

Genes potentially encoding 13 members of aquaporins (AQP0-12) have been identified in the human genome (Prat et al. 2011). Those 13 proteins are divided into three subgroups. The first group are the orthodox aquaporins, including AQP0,1,2,4,5,6 and 8 (Ishibashi et al. 2009). Although AQP6 and 8 are categorised in the same group based on primary sequence, AQP6 is permeated by anions (Yasui et al. 1999) and AQP8 is possibly permeated by water and urea (Ma et al. 1997). Members of the second group (aquaglyceroporins), which includes AQP3, 7, 9 and 10, are permeated by water to different extents as well as glycerol and possibly other small solutes (King et al. 2004). The third group is called superaquaporins, which includes AQP11 and AQP12. The members of this group are highly deviated from the rest and functional data for this group are limited. To date, evidence for the involvement of AQP11 in water transport (Ishibashi et al. 2009) and salivary gland development in mice

(Larsen et al. 2010) and involvement of AQP12 in controlling secretion of pancreatic fluid (Ohta et al. 2009) have been provided. Aquaporins have a very wide tissue distribution. They are expressed in many fluid transporting tissues, such as kidney tubules and glandular epithelia, as well as in non-fluid transporting tissues, e.g. adipose tissue (Verkman 2005). Aquaporins are also physiologically diverse, e.g. AQP4 has an important role in brain and corneal swelling (Verkman 2005) whereas AQP0 maintains eye- lens transparency (Ishibashi et al. 2009). As eight aquaporins are expressed in kidney, it seems they also play an essential role in kidney physiology (Ishibashi et al. 2009). For example, AQP1 and AQP2 are involved in urine concentration (King et al. 2001; Verkman 2005). AQP1 also plays role in increasing tumor cell migration through facilitated water transport and consequently increased angiogenesis (Verkman 2005; Monzani et al. 2009). Since aquaporins are implicated in the pathophysiology of a number of disorders of altered water homeostasis, such as brain edema, glaucoma, tumor growth (King et al. 2000; Frigeri et al. 2007) and neurodegenerative diseases (Foglio and Rodella 2010) they are potential targets for drugs modulating their function or expression. The human aquaporin characteristics and distribution are summarised in Table 1 below.

Aquaporin	Characteristic	Tissue distribution
AQP0	Low water transport	Eye lens
AQP1	High water transport	Red blood cell, brain, eye, lung, heart, kidney, salivary gland, muscle, liver, …
AQP2	High water transport	Kidney
AQP3	High water, high glycerol, moderate urea transport	Kidney, skin, eye, brain, heart, red blood cells, salivary gland
AQP4	High water transport	Brain, eye, heart, kidney, muscle
AQP5	High water transport	Eye, lung, salivary glands, …
AQP6	Low water transport, anions ($NO3^-$, Cl^-)	Kidney, brain
AQP7	High water, high glycerol, high urea, arsenite transport	Heart, kidney, testis, ovary
AQP8	High water transport	Liver, pancreas, testis, ovary, …
AQP9	Low water, high glycerol, high urea, arsenite transport	Liver, spleen, testis, ovary, leukocytes
AQP10	Low water, high glycerol, high urea	Gastro Intestinal tract
AQP11	High water transport	Brain, heart, liver, kidney, testis, ovary,…
AQP12	?	Pancreas

Table 1. The characteristics and distribution of human aquaporins
Compiled from (Ishibashi et al. 2009) and (King et al. 2004)

5.1.2. Plants

Aquaporins play a central role in plant water relations (Johansson et al. 2000; Maurel et al. 2008). Plant aquaporins are permeable to boron, hydrogen peroxide, CO_2, small alcohols, ammonia (Chaumont et al. 2005), arsenite and antimonite (Bienert et al. 2008) in addition to water. Therefore, they have also been linked to plant mineral nutrition and carbon and nitrogen fixation (Maurel et al. 2008). Plant aquaporins display a large isoform multiplicity, with 35 and 33 homologs in *Arabidopsis* and rice, respectively (Maurel et al. 2008). They are present in all subcellular compartments within secretary pathways (Maurel et al. 2008). Aquaporins in plants are divided into four subfamilies. The first subfamily is the plasma membrane intrinsic protein (PIP), the most abundant aquaporins in the plasma membrane, which cluster into two subgroups, PIP1 and PIP2. The second subfamily comprises of the tonoplast intrinsic proteins (TIPs) clustering into five subgroups (1 to 5), which are the most abundant aquaporins in the vacuolar membrane. The nodulin-26-like intrinsic membrane proteins (NIPs) form the third group and the fourth class comprises small intrinsic membrane proteins (SIPs) (Maurel et al. 2008).

5.1.3. Microorganisms

A wide range of potential physiological functions, including osmo-regulation and turgor pressure homeostasis, uptake of carbon and energy sources, export of metabolic end products, germination and transport of toxic metalloids have been described for microbial aquaporins (Hohmann S. et al. 2001). For instance, *E. coli* possesses one aquaporin (AqpZ) and one aquaglyceroporin (GlpF) (Borgnia and Agre 2001). AqpZ is involved in short- and long-term osmoregulatory responses and is required for rapid growth (Calamita 2000), whereas the aquaglyceroporin GlpF is involved in taking up glycerol as a carbon source (Heller et al. 1980; Lu et al. 2003). The aquaglyceroporin in the protozoan parasite *Leishmania* is involved in metalloid transport, such as arsenite and antimonite uptake (Gourbal et al. 2004). Moreover, the abundance of aquaporins in different microorganisms seems to vary greatly (Tanghe et al. 2006). Several yeast species possess only one aquaporin (*S. pombe*), whereas others have up to five (*A. nidulans*) (Pettersson et al. 2005). The genome of *S. cerevisiae* possesses *AQY1* and *AQY2* genes encoding for two orthodox aquaporins and *FPS1* and *YFLO54C* genes encoding for aquaglyceroporins (Pettersson et al. 2005). Interestingly, in most laboratory strains, there are spontaneous inactivating mutations in both *AQY1* and *AQY2* such that the gene products are not able to transport water across the plasma membrane, and hence are non-functional protein (Pettersson et al. 2005). Natural isolates

and less domesticated strains usually contain a functional Aqy1 but not Aqy2, suggesting that the presence of Aqy2 is not advantageous for yeast in such conditions (Carbrey et al. 2001; Tanghe et al. 2006), while Aqy1 must have some essential function for survival in nature (Tanghe et al. 2006; Will et al. 2010).

Aqy1 is a spore specific aquaporin and it is suggested to play a role in spore maturation, probably by controlling water outflow. Whereas the protein seems to be very poorly expressed in vegetative cells, its expression is stimulated during sporulation and is exclusively detected in spore membranes. Under conditions of rapid freezing, the spores lacking *AQY1* survive better, providing a functional test for active spore water channels, whereas under ambient conditions, a lack of Aqy1 decreases fitness. Studies have shown that the reduction in fitness is independent of germination conditions, and therefore it is believed that Aqy1 is important during spore formation rather than maintenance or germination (Sidoux-Walter et al. 2004).

The aquaporin Aqy2 is 88% identical to Aqy1, indicating recent gene duplication. On the other hand, expression of Aqy1 and Aqy2 is regulated differently suggesting functional specialisation (Pettersson et al. 2005). The Aqy2 protein is mainly located in the plasma membrane (Carbrey et al. 2001) and its expression is tightly regulated. The protein can only be detected in exponentially growing cells but not at all in resting cells (F. Sidoux-Walter and S. Hohmann, unpublished data), and the mRNA level diminishes upon nutrient depletion (Meyrial et al. 2001). It has been demonstrated that expression of Aqy2 is stimulated by the protein kinase A catalytic subunit Tpk2, which could potentially explain the observed growth- phase regulation (Robertson et al. 2000). Moreover, it has been shown that upon a shift to high osmolarity, expression of Aqy2 is diminished, but it reappears by returning to lower osmolarity. The down regulation accompanying osmotic shock seems to be partly dependent on the HOG pathway (F. Sidoux-Walter and S. Hohmann, unpublished data). This observation could indicate that Aqy2 plays a crucial role in allowing water efflux to control turgor during rapid growth or exposure to low osmolarity conditions (Hohmann and Mager 2003). It has further been shown that Aqy2 is involved in controlling cell surface properties, where its expression is controlled by osmoregulatory and morphogenic signalling pathways (Furukawa et al. 2009).

The product of *YFL054C* is about 35% identical to Fps1 for a stretch of 84 amino acids, although it shows more similarity to the products of both human AQP1 and yeast *AQY1* than it does to Fps1 (Coury et al. 1999). *YFL054* homologues exist in other yeasts and some filamentous fungi, and a *YFL054*- like protein is the only aquaporin found in the fission yeast *S. pombe* (Pettersson et al. 2005). The physiological role for *YFL054* in *S. cerevisiae* is still unknown, but it has been shown that *YFL054* mediates passive diffusion of glycerol in the presence of ethanol (Oliveira et al. 2003). Under normal conditions however, *YFL054* does not seem to mediate glycerol entry since *fps1⊿* and *fps1⊿yfl054⊿* show identical glycerol

uptake. It has also been reported that deletion of either *FPS1* or *YFL054* causes enhanced passive diffusion of ethanol (Oliveira et al. 2003), which could be due to altered membrane composition.
The yeast Fps1 protein is an aquaglyceroporin that functions as a channel mediating glycerol transport in response to changes in extracellular osmolarity. Fps1 is the main focus of this study and will be discussed in further detail later.

5.2. Structural features

The aquaporins are small, hydrophobic, intrinsic membrane proteins. Sequence analysis has revealed a strong similarity between the two halves of the molecule, indicating a tandem, intergenic duplication (Preston and Agre 1991; Krane and Kishore 2003). The repeats are assumed to be oriented at 180^0 to each other as the amino- and carboxy- termini are intracellular (Kozono et al. 2002). The walls of the pore are formed by six transmembrane spanning domains connected with five loops (A-E), of which loops A, E and C are extracellular and loops B and D are intracellular (Krane and Kishore 2003; Gonen and Walz 2006). A seventh transmembrane region is created by the cytoplasmic loop B and extracellular loop E, which are short helices (hemipores) that enter but do not span the bilayer (Hedfalk et al. 2006). The two hemipores fold into the membrane from opposite surfaces of the bilayer and interact with one another. Each hemipore contains the highly conserved signature motif NPA (Asn-Pro-Ala) placed at the centre of the channel (Kozono et al. 2002; Gonen and Walz 2006; Tornroth-Horsefield et al. 2010). An additional structural feature, located close to the extracellular end of the pore, conserved throughout the aquaporin family, is aromatic/arginine (ar/R) (Hedfalk et al. 2006; Mitani-Ueno et al. 2011). Although each AQP monomer is a functional water pore, they form homotetramer (Agre and Kozono 2003).

5.3. Substrate selectivity and specificity

Members of the aquaporin family facilitate highly efficient and specific passive diffusion of water and small, uncharged solutes across the membrane (de Groot and Grubmuller 2001). The human aquaporin AQP1 permeates water across the membrane at the rate of $3x\ 10^9$/s, per channel (Zeidel et al. 1992). Despite the extremely high transport rate, these channels maintain stringent selectivity. For instance, proton permeation is fully blocked even though it is known that protons can be conducted well by hydrogen-bonded water chains (de Groot and Grubmuller 2001). Blocking of proton permeation by aquaporins is fundamental for maintaining the electrochemical gradient across cellular and subcellular membranes (de

Groot et al. 2003). Moreover, while the bacterial glycerol facilitator GlpF facilitates permeation of glycerol and polyhydric alcohols, it hinders passage of water molecules, which are significantly smaller. Some aquaporins, such as the aquaporin from *Lactococcous lactis* and PfAQP from *plasmodium falciparum*, display a mixed function and transport both water and glycerol (Froger et al. 2001; Hansen et al. 2002). It has been proposed that aquaporins act as two-step filters and that the NPA and ar/R regions of aquaporins exert a large effect on the substrate specificity and selectivity of aquaporins (Mitani-Ueno et al. 2011). Simulations have revealed that water molecules maintain single- file passage through the channel, yet a reorientation occurs across the NPA motifs, which is believed to be due to the electrostatic field centred on the NPA motifs. This electrostatic barrier seems to be the main reason for proton exclusion (Tajkhorshid et al. 2002; de Groot et al. 2003; Hedfalk et al. 2006). The aromatic/arginine (ar/ R), on the other hand, is an important constriction region (Hedfalk et al. 2006; Mitani-Ueno et al. 2011). It has been proposed that the steric effects that constrict or widen the pore diameter near the ar/R region and amino acid substitutions that create preferences for hydrophobic or hydrophilic substrates are the major factors tuning pore selectivity (Hedfalk et al. 2006).

5.3.1. Transport of ions

With very few exceptions, ion permeability is not a general feature of aquaporins. Theoretically, a dehydrated ion is small enough to pass through the pore. However, the arrangements of the carbonyl oxygen atoms of each side of the pore, only allow partial dehydration of the ions, thereby inhibiting their passage through the pore (Kozono et al. 2002). One exception is AQP6, which is functionally distinct from other aquaporins. AQP6 exhibits low basal water permeability when expressed in *Xenopus laevis* oocytes. The water permeability of AQP6 oocytes rapidly increases up to tenfold, when treated with the known water channel inhibitor Hg^{2+} or pH less than 5.5, and is accompanied by ion conductance (Yasui et al. 1999). AQP6 exhibits anion permeation with marked specificity for nitrate anions (Ikeda et al. 2002). AQP1 apparently not only mediates water flux but also serves as a cGMP-gated ion channel with non-selective cationic permeability (Anthony et al. 2000; Boassa et al. 2006).

5.3.2. Transport of gases

The lipid phase of all biological membranes is thought to be freely permeable to gases and gaseous molecules diffusing through the membrane, although this does not explain their rapid physiological action. Aquaporins are often expressed in tissues, where gas transport is

essential, suggesting that perhaps these channels conduct physiologically important gases in addition to water (Herrera and Garvin 2011). For instance, AQP1 appears to transport gases such as CO_2 (Cooper and Boron 1998) and NH_3 (Nakhoul et al. 2001), although the biological significance of AQP1- dependent gas transport is still under debate (Verkman 2005). Several limitations make it difficult to determine the full importance of AQP1 to CO_2 permeability, such as the possible existence of an as yet unidentified CO_2 channel, which would mask this contribution (Herrera and Garvin 2011). Similar CO_2 transport rates have been shown in tobacco aquaglyceroporin NtAQP1, which shares a high sequence similarity to human AQP1 in the pore region (Uehlein et al. 2003). In addition, NO, which has an important role in blood pressure regulation, can be transported through human AQP1 across the cell membrane (Herrera et al. 2006).

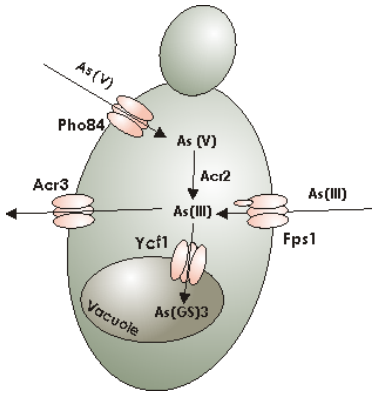
5.3.3. Transport of metalloids (current study)

Arsenic is a metalloid that is widely distributed in the earth's crust and occurs in four oxidation states +5, +3, 0 and -3 but predominantly exists as either pentavalent (As(V)) or trivalent (As (III)) species, of which the latter is considered to be the more toxic form (Bhattacharjee et al. 2009). Beside its natural occurrence, arsenic is applied in commonly used herbicides, insecticides, dyes and wood preservatives. Humans are usually exposed to arsenic through industrial activities, such as mining and coal burning, or by drinking water that has percolated through arsenic- rich soil (Liu et al. 2001; Waxman and Anderson 2001). Arsenic is ranked as first on the U.S. Department of Health and Human Services 'superfund priority list of hazardous substances. Long-term exposure to arsenic causes a number of human diseases, including diabetes (Lai et al. 1994; Tseng et al. 2000) kidney, and cardiovascular diseases, and it is associated with liver, lung, skin and bladder cancers (Chappell 1998; Tamas and Wysocki 2001). Despite the toxicity of arsenic, it has a long history of usage for medical purposes. In particular, it is used in the treatment of diseases caused by protozoan parasites, such as *Trypanosoma* and *Leishmania*. In the second stage of sleeping sickness or Human African Trypanosomiasis (HAT), treatment is exclusively dependent on the arsenical compound melarsoprol (Nok 2003). Arsenic is also an active ingredient in the chemotherapeutic drug Trisenox, which is used for the treatment of acute promyelocytic leukaemia (Barrett 2003; Douer et al. 2003). Application of arsenical pastes for skin cancer and arsenous acid for hypertension, heartburn and chronic rheumatism treatments have also been described (Aronson 1994). The major arsenite species in solution is $As(OH)_3$, which is an inorganic molecular mimic of glycerol. Structural, thermodynamic and electrostatic comparisons of $As(OH)_3$ and $Sb(OH)_3$ at physiological pH, have shown that both compounds have a slightly smaller volume but similar conformation and charge distribution compared to glycerol (Porquet and Filella 2007). More recently, some aquaglyceroporins were

reported to transport arsenite and antimonite (Bhattacharjee et al. 2009). The glycerol facilitator GlpF in *E.coli* was the first discovered entry route for antimonite (Sanders et al. 1997). In mammalian cells, AQP9 and to a lesser extent AQP7 are the main routes of arsenite uptake (Liu et al. 2002).

In a current study on plant aquaporins (unpublished data), applying heterologous expression in yeast, we showed that *Arabidopsis thaliana* aquaporin AtTIP4;1 mediates arsenite uptake. Yeast cells are highly sophisticated equipped with several arsenic detoxification mechanisms (Figure 4). These mechanisms involve arsenate reductase Acr2, plasma membrane localised arsenite extrusion transporter Acr3 and vacuolar ABC transporter Ycf1 (Tamas and Wysocki 2001) thus, they are relatively resistant to arsenite. Particularly, deletion of *FPS1* that encodes the main entry route of arsenite in yeast (Wysocki et al. 2001) confers a high level of arsenite resistance. In this strain, only far higher arsenite concentrations cause toxicity (Wysocki et al. 2001). Such high arsenite concentrations rarely occur in plant environment and theoretically could force the membrane to become permeable to arsenite upon high concentration gradient. Thus, we used the *fps1⊿acr3⊿ycf1⊿* mutant, which is both impaired in aquaglyceroporin as well as in detoxification mechanisms, hence highly sensitive to arsenite (Wysocki et al. 2001).

Figure 4. Mechanisms involved in arsenic transport and tolerance in *S. cerevisiae*.
Pentavalent arsenate enters cells via phosphate transporters such as Pho84 (Bun-ya et al. 1996). In the cytosol, arsenate is then reduced to arsenite by the arsenate reductase Acr2 (Mukhopadhyay et al. 2000). The uptake of trivalent arsenite is mediated by the glycerol channel Fps1 (Wysocki et al. 2001). The arsenite is then either immediately extruded from the cell by Acr3 (Wysocki et al. 1997) or conjugates to glutathione and sequesters to the vacuole through the ABC transporter Ycf1 (Ghosh et al. 1999).



To test whether plant aquaporin AtTIP4;1 transports As(III), it was expressed in the *fps1⊿acr3⊿ycf1⊿* yeast strain and growth upon arsenite exposure as well as arsenite uptake were monitored. We observed that *fps1⊿acr3⊿ycf1⊿* cells expressing rAQP9 (positive control) (Bienert et al. 2008) and AtTIP4;1 displayed increased As(III) sensitivity and arsenite uptake compared to the empty vector control indicating that AtTIP4;1 mediates arsenite influx into yeast (data not shown).

Moreover, some aquaglyceroporins are reported to be bidirectional channels involved in arsenic detoxification. For example, upon exposure to arsenate, in legume symbiont *Sinorhizobium meliloti*, arsenate is reduced to arsenite, which flows out of the cell upon downhill gradient through aquaglyceroporin AqpS (Yang et al. 2005). Certain NIPs from *Arabidopsis* can improve growth in the presence of arsenate, strongly suggesting that they

mediate As(III) efflux and supporting the notion that these proteins act as bidirectional channels (Bienert et al. 2008). Because of the biological importance of arsenic compounds, it is essential to gain a better understanding of the function and regulation of uptake pathways, the proteins that modulate the activity of such pathways as well as the systems mediating detoxification and tolerance at the molecular level. The involvement of *S. cerevisiae* Fps1 in arsenite uptake and detoxification will be discussed in further detail later.

5.4. Regulation

Aquaporins play vital roles in highly diverse and important physiological processes in all kingdoms of life. Hence, understanding the mechanisms of regulation of these proteins is imperative. Aquaporins can be regulated either at the transcriptional level or post-translationally via a number of different mechanisms. For instance, phosphorylation of conserved serine or threonine residues is suggested to play a role in both gating and trafficking of aquaporins (King et al. 2004; Woo et al. 2008). Some aquaporins are regulated by changes in pH (Nemeth-Cahalan et al. 2004), divalent cation concentrations (Nemeth-Cahalan et al. 2004) or osmolarity (Tamas et al. 2003).

5.4.1. Expression

Many aquaporins are subject to transcriptional- level rather than short- term regulation (Verkman 2005). For example, monitoring the expression pattern of all 35 *A. thaliana* aquaporins has revealed that most PIPs and some TIPs have a high level of expression, whereas NIPs are present at a much lower level. PIPs are generally transcriptionally downregulated upon gradual drought stress in leaves (Alexandersson et al. 2005). In mammals, it has been shown that the AQP3 gene expression is regulated by a transcriptional regulator Foxa2, which is regulated by insulin (Higuchi et al. 2007). Studies have also shown that AQP7 mRNA expression is tightly regulated by insulin at the transcriptional level (Fruhbeck 2005). In rats, AQP1 can be found in the lungs shortly before birth and its level increases noticeably during the perinatal period. Corticosteroids induce AQP1 expression in both fetus and adult lungs (King et al. 2004). In *S. cerevisiae*, *AQY1* is abundantly expressed when diploid cells are shifted to sporulation conditions, whereas *AQY2* is expressed in exponentially growing cells (Pettersson et al. 2005).

5.4.2. pH

Aquaporin-mediated water transport is modified by pH changes. For instance, at least three mammalian aquaporins (AQP0, 3 and 6) seem to be regulated by pH (Engel et al. 2000). The water permeability of AQP0, the water channel of lens- fibre cells, is regulated by pH, Zn^{2+}, Ni^{2+} and Ca^{2+}. In the *Xenopus* oocyte expression system, shifting the pH from 7.4 to 6.5 increases AQP0 water permeability, via a process involving an extracellular histidine (His40) in the A loop (Nemeth-Cahalan and Hall 2000). AQP3 is another mammalian aquaporin that is expressed in the basal lateral membranes of collecting duct cells in the kidney. AQP3 is reportedly permeated by water and glycerol at neutral pH, but the channel gates shut at pH lower than 6. His53, Tyr124, and His154 have been shown to be involved in the regulation of AQP3 by extracellular pH (Zeuthen and Klaerke 1999; Zelenina et al. 2003). Regulation of aquaporin permeability by pH occurs in plants as well. For instance, the *A. thaliana* water channels AtPIP2;1, AtPIP2;2, AtPIP2;3 and AtPIP1;2 close upon a shift of the cytosolic pH from 7 to 6 when studied in *Xenopus* oocytes. The residue primarily responsible for pH-mediated gating of AtPIP2;2 was shown to be His 197, which is localised in intracellular loop D. Substitution of His 197 by an alanine residue reduces the effect of cytosol acidification (Tournaire-Roux et al. 2003).

5.4.3. Trafficking

Trafficking is one of the mechanisms, by which aquaporins are regulated, whereby they are shuttled from intracellular storage sites to the plasma membrane (Tornroth-Horsefield et al. 2010). For instance, in *A. thaliana* a specific phosphorylated site in the C terminus of AtPIP2;1 determines trafficking of this aquaporin in resting conditions and in response to an NaCl treatment (Prak et al. 2008). AQP2 is a mammalian aquaporin expressed in the principal cells of connecting tubules and collecting ducts of kidney and plays a critical role in the concentration of urine. AQP2 is localised in the intracellular compartment and is translocated to the cell surface upon anti-diuretic hormone stimulation. The translocation process is regulated through phosphorylation of AQP2 by protein kinase A. The actin cytoskeleton plays an important role in the trafficking of AQP2 (Takata et al. 2008).

5.4.4. Phosphorylation

Reversible protein phosphorylation is one of the most important and well-studied post-translational modifications. Phosphorylation of conserved serine or threonine residues is

suggested to play a role in both gating and trafficking of eukaryotic aquaporins (Tornroth-Horsefield et al. 2010). It has been shown that the transport activity of PM28A, an aquaporin in the spinach leaf plasma membrane, is regulated by phosphorylation of two serine residues: Ser 115 in the first cytoplasmic loop and Ser 274 in the C-terminal region (Johansson et al. 1998). AQP4, the predominant aquaporin found in brain, can be phosphorylated by PKC (protein kinase C) on serine 180. Since observations of GFP- labelled AQP4 did not imply regulation by trafficking, it was concluded that the effect of serine 180 phosphorylation was to mediate water permeability by gating (Zelenina et al. 2002). A reduction in AQP4 water permeability following its activation by PKC has also been reported in renal epithelial cells (King et al. 2004). We have shown that in *S. cerevisiae*, the aquaglyceroporin Fps1 is phosphorylated on threonine 231 by the MAPK Hog1 and this phosphorylation critically affects the activity of the protein (Paper I). There is also a report of phosphorylation and thereby destabilisation of Fps1 by Hog1 upon acetic acid exposure, which subsequently down regulates the conductance of acetic acid through Fps1 (Mollapour and Piper 2007).

5.4.5. Divalent cations

The divalent cation concentration can directly influence aquaporin gating (Tornroth-Horsefield et al. 2010). In the aquaporin of the beet (Beta Vulgaris) storage root, the divalent cation Ca^{2+} can finely down- or up- regulate water channel activity (Alleva et al. 2006). It has also been shown that lowering the external Ca^{2+} increases AQP0 water permeability (Nemeth-Cahalan and Hall 2000).

5.4.6. Osmotic changes

Changes in osmolarity can directly influence the transport activity of aquaporins. One example is Fps1, which is rapidly regulated by osmotic changes. Within seconds following a hyperosmotic shock, glycerol efflux through Fps1 is diminished to enable accumulation of glycerol. On the other hand, upon hypo osmotic condition, Fps1 re-activation/ opening within a similar time-scale ensures release of excess glycerol and proper turgor pressure (Tamas et al. 1999; Tamas et al. 2003).

5.5. Fps1 a glycerol channel in yeast

5.5.1. Discovery

FPS1 (*fdp1* suppressor), was first discovered as a multicopy suppressor of defective growth of the *fdp1* mutant on fermentable sugars (Van Aelst et al. 1991). The growth phenotypes of *fdp1* mutants were soon after shown to be due to an imbalance in glycolysis and could be rescued by additional deletion of gene encoding the main hexokinase, *HXK2* (Hohmann et al. 1993). Luyten et al.(1995) later showed that overexpression of *FPS1* leads to enhanced glycerol production and export, which in turn relieves the block in glycolysis of this mutant. It was also demonstrated that mutants lacking Fps1 accumulate more intracellular glycerol, indicating that Fps1 is involved in glycerol efflux. Glycerol-uptake experiments showed that the permeability of the yeast plasma membrane to glycerol consists of an Fps1-independent component, probably due to simple diffusion, and an Fps1-dependent component, corresponding to facilitated diffusion. Thus, it was concluded that Fps1 is a regulated yeast glycerol facilitator, which controls glycerol production and cytosolic concentration and may have additional functions (Luyten et al. 1995).

5.5.2. Structural features

Fps1 is an atypical aquaglyceroporin. Although, it was originally believed that aquaglyceroporins function as monomers (Lagree et al. 1998), it has now been shown that Fps1 and other aquaglyceroporins function as a homotetramer (Beese-Sims et al. 2011). The highly conserved NPA (Asn- Pro- Ala) motifs in the B and E loops are replaced by NPS (Asn- Pro- Ser) and NLA (Asn-Leu- Ala), respectively (Bill et al. 2001). In addition, while most of the members of the aquaporin family are less than 300 amino acids long (Hedfalk et al. 2004), Fps1 consists of 669 amino acids (Tamas et al. 1999). The larger size is mainly due to the extended N- and C-terminal domains, and the poor conservation in these domains suggests the involvement in regulation rather than function (Hedfalk et al. 2004). Fps1 has long, distinct N- and C-terminal extensions of 255 and 139 amino acids, respectively (Tamas et al. 2003). Deletion of the hydrophilic N-terminal domain or mutations in specific residues within this domain results in constitutive release of glycerol due to an unregulated channel, which in turn causes sensitivity to high osmolarity conditions and glycerol overproduction (Tamas et al. 1999; Tamas et al. 2003). The critical part of N-terminal regulatory domain is narrowed down to 12 amino acids located between residues 225 and 236

([225]LYQNPQTPTVLP[236]), close to the first transmembrane domain. The sequence of the regulatory domain and its position are perfectly conserved in orthologs from other yeast species. The role of this regulatory domain is to restrict transport through Fps1. Additional mutagenesis analysis has revealed that residues Leu-225, Asn-228, Gln-230, Thr-231 and Pro-232 are of particular importance for channel control (Tamas et al. 2003).
Truncations in the C terminus of Fps1 also confer an osmosensitive phenotype. The observed phenotype is a result of inefficient accumulation of intracellular glycerol under hyperosmotic conditions. Hence, it appears that the Fps1 C terminus has a similar function to the N terminus in restricting glycerol transport through Fps1. A sequence of 12 amino acids, residues 535–546 ([535]HESPVNWSLPVY[546]), close to the sixth transmembrane domain has been identified as important for controlling Fps1 function (Hedfalk et al. 2004).
Some of the residues underlined above will be discussed in further detail in subsequent parts of the thesis.

5.5.3. Physiological role

5.5.3.1. Osmohomeostasis and glycerol transport

The physiological role of Fps1 in osmohomeostasis is to control the intracellular level of glycerol. Processes to counteract water loss from the cell are central for the adaptation of yeast cells to hyperosmotic conditions. This is achieved by accumulating a high intracellular pool of glycerol acting as compatible solute. Upon hyperosmotic conditions, Fps1 is rapidly inactivated/ closed to ensure retention and accumulation of glycerol (Tamas et al. 1999). The *fps1*Δ mutant adapts to hyperosmotic shock comparably to WT cells, whereas mutants expressing a constitutively open Fps1 (Fps1-Δ1) or point mutants that render Fps1 an unregulated channel cannot retain glycerol, and therefore are hyper-osmosensitive (Tamas et al. 2003). Once the cell has accumulated enough glycerol or it is shifted to lower external osmolarity, Fps1 is activated/ opens again to release glycerol and decrease turgor pressure. Fast release of glycerol is important for survival, as mutants lacking Fps1 only poorly withstand hypo-osmotic shock (Tamas and Hohmann 2003).

5.5.3.2. Anaerobic growth

Under anaerobic growth conditions, glycerol production is required to reoxidize excess NADH (Albers et al. 1996; Ansell et al. 1997). Accumulation of glycerol under such conditions will lead to an increase in turgor pressure if not efficiently exported. Therefore, Fps1 function is vital in such conditions for efficient glycerol export. Mutants lacking Fps1

show a higher intracellular glycerol level and grow poorly under anaerobic conditions (Tamas et al. 1999).

5.5.3.3. Cell fusion of yeast mating cells

As mating of two haploid yeast cells of opposite mating type progresses, the cells reorganise their cell walls to allow the fusion event to occur (Philips and Herskowitz 1997). Fusion is regulated both temporally and spatially; the cell wall is only degraded after cell- cell contact and only in the region of the cell-cell contact. The *fps1*⊿ cells sense osmotically imbalanced conditions due to increased intracellular glycerol levels and respond by preventing cell wall breakdown; hence, *fps1*⊿ mutants are defective in cell fusion. The defect of *fps1*⊿ cells in mating can be suppressed by providing extracellular sorbitol as an osmotic stabiliser or by reducing intracellular glycerol production. These observations provide evidence that the osmotic state of the cell regulated by Fps1, can affect cell fusion during mating (Philips and Herskowitz 1997).

5.5.3.4. Membrane composition

To investigate possible differences in general permeability of WT and *fps1*⊿ cells, ethanol-enhanced proton diffusion constants have been determined. The results showed that WT has a greater permeability for ethanol- enhanced proton diffusion compared to the *fps1*⊿ mutant, indicating that Fps1 probably affects membrane structure or composition. Further investigation of the cellular lipid composition in a *FPS1* deletion strain revealed that the phospholipid and glycolipid fractions of the total lipid of the *fps1*⊿ mutant are 25% lower and 62% higher, respectively, than in the WT, suggesting that the reduction in membrane permeability may be connected to the cellular lipid composition (Sutherland et al. 1997). Similarly the relationship between *fps1*⊿, glycerol efflux and membrane ergosterol content has been studied (Toh et al. 2001). Ergosterol is the most abundant sterol in yeast membranes (Zinser et al. 1993) and plays an important role in membrane structure, thereby influencing permeability to solutes. The ergosterol content of the plasma membranes of the *fps1*⊿ strain has been shown to be approximately 18% at the whole cell level and 26% at the plasma membrane level lower than that of the WT strain. Ergosterol supplementation facilitated the flux of glycerol across the plasma membrane of yeast cells and also partially suppressed the hypoosmotic sensitivity of *fps1*⊿ mutant (Toh et al. 2001). Although these observations indicate that Fps1 affects membrane/cellular lipid composition, the exact mechanism remains to be established.

5.5.3.5. Freeze tolerance

Under conditions of slow freezing, intracellular water has a sufficient amount of time to diffuse out of the cell through plasma membrane, and hence water permeability of the plasma membrane is not rate limiting. The membrane permeability for water drops substantially with drop in temperature, therefore membrane water permeability becomes limiting at the conditions of rapid freezing. Cells prevent intracellular ice crystal formation and consequent cell damage through aquaporin mediated water outflow (Tanghe et al. 2006). Whereas the presence of aquaporins increases freeze tolerance, the opposite effect has been reported for the aquaglyceroporin Fps1 (Izawa et al. 2004a). Glycerol is well known as a cryoprotectant (Siderius et al. 2000). Cells with higher intracellular glycerol levels acquire freeze tolerance and retain high leavening ability in dough after freeze storage (Izawa et al. 2004b). Accordingly, the *fps1⊿* mutant, which accumulates high intracellular glycerol, shows high freeze tolerance. Hence, it is a useful strain for developing better frozen-dough with commercial advantages (Izawa et al. 2004a).

5.5.3.6. Trivalent metalloid uptake and efflux

Fps1 mediates influx of metalloids arsenite and antimonite in *S. cerevisiae*. The *fps1⊿* mutants are resistant to these metalloids. Same tolerance is exhibited by WT cells exposed to hyper osmotic conditions, when Fps1 is inactive. Accordingly, yeast transformants expressing constitutively open Fps1 are highly sensitive to both arsenite and antimonite (Wysocki et al. 2001). A recent study has provided evidence that Fps1 is an arsenic bidirectional channel. Therefore, a role in arsenic detoxification has been suggested for Fps1. Overexpression of Fps1 restores the sensitive growth phenotype upon arsenite exposure. The ability of Fps1 to mediate As(III) export has further been demonstrated in efflux assays (Maciaszczyk-Dziubinska et al. 2010).

5.5.3.7. Acetic acid uptake

Fps1 facilitates the entry of uncharged, undissociated acetic acid into the cell. Loss of Fps1 essentially eliminates acetic acid accumulation in cells at low pH. Cells exposed to the same concentration of acetic acid at neutral pH, when the acetic acid in the medium is almost completely dissociated and in the form of acetate anion, exhibit decreases cellular accumulation of acetate. It has been suggested that the Fps1 pore is too small to accommodate the acetate anion in its dissociated form (Mollapour and Piper 2007).

5.5.4. Regulation

5.5.4.1. The N-terminal regulatory domain and Hog1 (Paper I)

As described earlier, arsenic is broadly dispersed in nature and all organisms possess regulatory mechanisms to avoid toxicity and obtain tolerance. The activity of metal influx and detoxification systems is controlled by signalling proteins and transcriptional regulators. In mammals, As(III) activates the MAPK p38 (Cavigelli et al. 1996) and in *Schizosaccharomyces pombe* the MAPK Sty1 (Rodriguez-Gabriel and Russell 2005), which both are Hog1 homologues. In Paper I, we described a mechanism whereby Hog1 is phosphorylated upon arsenite exposure and mediates arsenite tolerance by affecting arsenite influx through Fps1. Arsenite uptake was higher in the *hog1⊿* mutant compared to WT, whereas the *hog1⊿fps1⊿* mutant exhibited a small amount of arsenite influx (Paper I, Figure 6B) and was as As(III) resistant as *fps1⊿* (Paper I, Figure 6A).

A conserved MAPK phosphorylation site (T231) within the N- terminal regulatory domain has been shown to play critical role in Fps1 transport activity. Mutation of threonine to alanine at this residue (T231A) leads to an unregulated channel that is comparable to that observed upon deletion of the N-terminal domain (Fps1- Δ1) (Tamas et al. 2003). In this study, we provided evidence that Fps1 is phosphorylated *in vivo* (Paper I, Figure 7A) on residue T231 in the N-terminal regulatory domain (Paper I, Figure 7B) and expression of Fps1-T231A clearly renders cells sensitive to As(III) (Paper I, Figure 7C). We also showed that Hog1 affects Fps1 phosphorylation *in vivo* (Paper I, Figure 7E) and Hog1 phosphorylates Fps1 on T231 *in vitro* (Paper I, Figure 7D). We concluded that Hog1 inhibits Fps1 activity in the presence of arsenite and under basal conditions, most likely by phosphorylating Fps1 on T231(Figure 5). This finding is to the best of our knowledge the first report of a MAPK modulating aquaporin/ aquaglyceroporin activity.

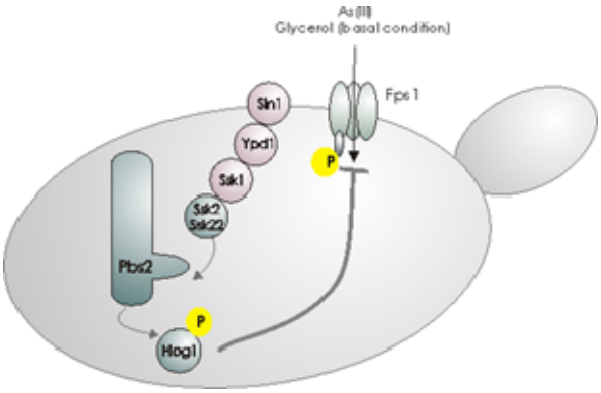


Figure 5. Hog1 is phosphorylated in response to arsenite. As(III) activation of Hog1 occurs through the Sln1- Ssk1 branch of the HOG pathway. Hog1 negatively modulates Fps1 transport activity under basal growth conditions as well as arsenite stress, probably by phosphorylating the residue T231 within the N-terminal domain of Fps1.

5.5.4.2. The C-terminal regulatory domain and Slt2 (Paper II)

We had previously suggested that other kinase(s) might be involved in controlling Fps1, as deletion of *HOG1* did not fully abolish *in vivo* phosphorylation of Fps1. Moreover, the observed increase in Fps1 phosphorylation levels in response to As(III) and NaCl treatments was independent of Hog1 (Paper I). In Paper II, we demonstrated that the MAPK Slt2 affects the transport activity of Fps1 (Figure 6). As MIP channels have a symmetrical topology and the first and the second half of the proteins share sequence homology, it was expected that the transport function of MIP channels would be bidirectional, probably with the same efficiency (Tamas et al. 1999). An increasing number of aquaglyceroporins have been reported that depending on the direction of the concentration gradient, catalyse bidirectional movement of metalloids. For instance, it has been shown that human AQP9 plays a crucial role in the detoxification of arsenic metabolites by facilitating efflux from the cells (McDermott et al. 2010). Recently, it was also demonstrated that Fps1 is a bidirectional channel and that overexpression of *FPS1* increases arsenite tolerance, suggesting a physiological role for Fps1 in arsenic detoxification. Here, we further demonstrated that whilst overexpression of *FPS1* clearly improves arsenite tolerance in WT cells, it neither confers tolerance in the *slt2*⊿ deletion mutant nor in the *bck1*⊿ (Paper II, Figure 2).
This observation suggests that Slt2 plays a crucial role in arsenite detoxification upon overexpression of *FPS1*. As previously reported, the contribution of Fps1 to arsenite export upon overexpression is somewhat difficult to capture in WT due to the on-going export of As(III) through the arsenite export pump Acr3 (Maciaszczyk-Dziubinska et al. 2010). Therefore, we employed deletion mutants impaired in *ACR3* function. Our phenotypic analysis clearly established that the *acr3*⊿*slt2*⊿ double deletion mutant exhibited additive sensitivity compared to the *acr3*⊿ single mutant, suggesting that Acr3 mediates As (III) efflux independently of Slt2 (Paper II, Figure 5A). Furthermore, consistent with the growth data, transport assays showed that the presence of multiple copies of *FPS1* resulted in a significant decrease in As(III) accumulation in the *acr3*⊿ mutant, whereas the ability of Fps1 to facilitate As(III) export in *acr3*⊿*slt2*⊿ was impaired (Paper II, Figure 5B). The transport data clearly indicated that Slt2 mediates arsenite detoxification and tolerance by mediating As(III) efflux through Fps1. By analysing the results of the growth tests and transport assays, we identified a residue (S537) within the C-terminal regulatory domain of Fps1, which may play an important role in Fps1 efflux activity (Paper II, Figure 6).
The most straightforward explanation for the results is that Slt2 activates Fps1 by phosphorylating residue S537 within the C-terminal regulatory domain of Fps1. Further investigations of the Fps1 and slt2 physical interaction and Fps1 phosphorylation by Slt2 are currently under way. Using an M-TRACK protein proximity assay, Gustav Ammerer and

colleagues have preliminarily shown that Fps1 and Slt2 physically interact (unpublished data). The preliminary data obtained from our co-immunoprecipitation (Co-IP) study also confirmed a physical interaction between Fps1 and Slt2 (data not shown).

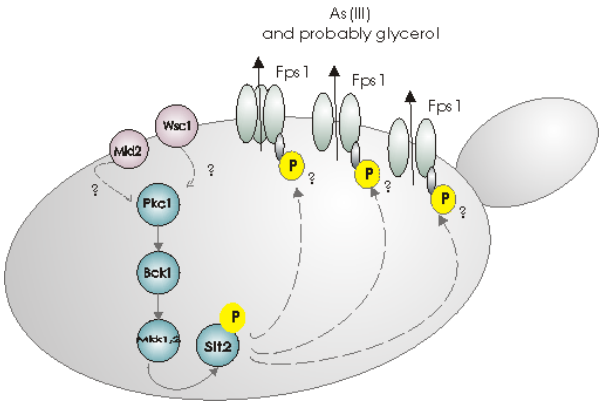


Figure 6 Slt2 is phosphorylated in response to arsenite in a Bck1 dependent manner. Slt2 stimulates arsenite efflux upon *FPS1* overexpression. The residue S537 within the C-terminal regulatory domain of Fps1 plays a crucial role in arsenite efflux. A plausible mechanism, where Slt2 physically interacts with Fps1 and phosphorylates the residue S537, is under investigation.

5.5.4.3. The transmembrane core (Paper III)

Most studies on fungal aquaporins have been conducted on the yeast *S. cerevisiae.* Although, Fps1- like proteins are present in many other fungi, among which one can point out *Ashbya gossypii*. The Fps1 in *A. gossypii* (AgFps1) is much smaller than in *S. cerevisiae* (ScFps1) (476aa compared to 669aa, respectively) (Tamas et al. 1999; Pettersson et al. 2005). The fact that *A. gossypii* accumulates glycerol upon osmotic stress (Forster et al. 1998) and our data demonstrating that AgFps1 in *A. gossypii* mediates transport of both glycerol and arsenite (Paper III, Figure1) suggest that AgFps1 in *A. gossypii* functions as an aquaglyceroporin and perhaps plays rather a similar role to ScFps1 in *S. cerevisiae*. We further showed that although expression of AgFps1 in *S. cerevisiae fps1*Δ demonstrates a survival advantage upon hypoosmotic stress, and thus AgFps1 is a functional channel in *S.cerevisiae* (Paper III, Figure 2A), it behaves as hyperactive, conferring osmo sensitive phenotypes (Paper III, Figure 2B). These phenotypes are comparable to that seen for cells expressing a point mutant in the N-terminal of Fps1, Fps1-N228A, which is known to behave as a hyperactive channel (Tamas et al. 2003) . As AgFps1 exhibits highly conserved but not identical N- and C- terminal regulatory domains to ScFps1 (Paper III, Supplemental Figure 1), we investigated whether the differences in the termini regulatory sequences of the two proteins explain AgFps1 hyperactivity. Therefore, we considered AgFps1 as a suitable Fps1 ortholog to study in comparison with ScFps1, to reveal the mechanisms behind the regulation of the Fps1 channel. To this end, we exchanged the N- and C-terminal extensions of AgFps1 for the corresponding extensions in ScFps1, and *vice versa* (Paper III, Figure 3A). Most of the constructs were located in fractions containing plasma membrane and were functional as

restored growth upon hypoosmotic condition (Paper III, Figure 3B). The constructs with the AgFps1 backbone behaved as wild type AgFps1 in response to hyperosmotic conditions and exhibited an osmosensitive phenotype (Paper III, Figure 3D). In contrast, cells expressing ScFps1 chimeras (with termini from AgFps1) showed no osmosensitivity and similar behaviour to wild type ScFps1 (Paper III, Figure 3D). Thus, we concluded that in addition to the N- and C- terminal domains that play a critical role in Fps1 activation/opening and inactivation/closure (Tamas et al. 2003; Hedfalk et al. 2004), the transmembrane core of the protein also participates in the regulation of transport activity.

We further searched for intragenic suppressor mutants for the hyperactive phenotype of N228A, to gain a better understanding on how Fps1 transport activity is regulated (Tamas et al. 2003). We found a suppressor mutant G519S, located in the middle of transmembrane domain 6 (TMD6) in Fps1, in close proximity to TMD4. Cells expressing the double ScFps1-N228AG519S point mutants as well as the single mutant G519S behaved as wild type Fps1 and were able to survive osmotic shocks in both directions, indicating a functional and regulated aquaglyceroporin (Paper III, Figure 4A). The intracellular glycerol levels upon hyperosmotic stress (Paper III, Figure 4B) were in good accordance with the growth data, suggesting that the poor growth caused by N228A is perhaps due to poor glycerol accumulation and hyperactivity of the channel, whereas the suppressor mutation restored controlled glycerol flux through the protein. The transport data further confirmed that whereas cells expressing ScFps1- N228A exhibit a high transport rate, cells expressing ScFps1-N228AG519S and ScFps1-G519S transport much less glycerol, comparable to wild type (Paper III, Figure 4C). It seems that an additional mutation in the transmembrane core is able to cancel out the effect of a prior mutation in the N-terminal regulatory domain, suggesting that this domain alone is not enough to restrict ScFps1 glycerol transport. Interestingly, this glycine is well conserved among yeast Fps1 orthologs as well as among most aquaporins and aquaglyceroporins, from bacteria to humans (Paper III, Figure 5B). Hence, we substituted the same glycine in AgFps1 with serine (G382S) and tested for survival under high osmotic-stress. Whereas cells expressing the wild type AgFps1 exhibited an osmosensitive phenotype due to an inability to accumulate glycerol, cells expressing AgFps1G382S could survive both a hypo- and hyper-osmotic shock (Paper III, Figure 5C). This data again suggests that the mutation in the transmembrane core (TM6) is able to counteract the hyperactivity of the channel. Previous studies on GlpF (bacterial aquaglyceroporin) and PpAqy1 (*Pichia pastoris* yeast aquaporin), have shown that this particular glycine plays an important role in building up the helix and stabilising the whole structure by packing against TM4 and forming several van der Waals bonds. We speculated that when glycine is substituted for a serine, which is larger in volume and more polar, the TM4 might be pushed inwards, thereby changing the property of the pore, and consequently decreasing the transport rate. This speculation may explain why yeast can survive both hypo-

and hyper-osmotic shock when expressing the G519S (or G382S in *A. gossypii*) variant of Fps1. We assumed that deletion or mutation in N- or C- terminal regulatory domains and changes in phosphorylation status might lead to improper binding to the core, thereby affecting the tilting of the helices, which in turn would alter the transport activity. This notion needs further investigation (Figure 7).

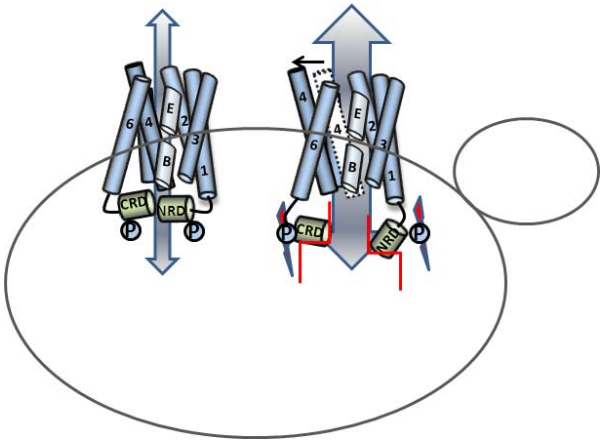


Figure 7. We propose a mechanism, whereby the flux through the channel is controlled by interplay between the transmembrane helices residues and the termini. Perhaps mutations in N- or C- regulatory domains, which lead to changes in phosphorylation status of the protein, will affect the termini and transmembrane core interplay and interrupt the regulated transport activity of Fps1 (Adapted from the courtesy by Dr. K. Lindkvist, University of Lund, Sweden).

5.5.4.4. Rgc1/ Rgc2 as positive regulators

A pair of proteins (Rgc1/ Rgc2) seem to function as positive regulators of Fps1 activity (Beese et al. 2009). The *rgc1/2* ⊿ mutant experiences severe cell wall stress and produces a fortified cell wall due to accumulation of glycerol and abnormally high turgor pressure, because of a defect in Fps1 activity. Normally, Fps1 resides as punctate spots at the plasma membrane (Tamas et al. 1999). As the number, location and intensity of Fps1 punctate spots were apparently not altered in the *rgc1/2*⊿ mutant, it was concluded that Rgc1/ Rgc2 affect Fps1 channel activity rather than its expression, folding or localisation. Since the *rgc1/2*⊿ mutant exhibited high levels of arsenite resistance, the authors concluded that Rgc1/2 function is required to open Fps1. Suppression of *hog1*⊿ arsenite hypersensitivity by *rgc1/2*⊿ mutations further suggested that a possible mechanism involved Hog1 promoting Fps1 closure by inhibiting the action of Rgc1/2. Rgc2 is phosphorylated under stress conditions that lead to inactivation of Fps1 in a partly Hog1 dependent manner. Apart from Hog1 negatively regulating Fps1 through phosphorylation on T231 (Paper I), this finding suggests a regulatory pathway from Hog1 to Rgc1/2 to Fps1, in which Rgc1 and Rgc2 are positive regulators of Fps1 channel activity and Hog1 inhibits Fps1 through inhibition of Rgc1/2 (Beese et al. 2009).

5.5.4.5. Endocytosis

Mollapour and Piper (2006) showed that upon exposure to acetic acid, Hog1 is activated. Hog1 activation occurs more rapidly at low pH, when the main form present is the undissociated acid, which is able to enter the cell. Hog1 activation does not lead to stimulation of *GPD1* gene expression or to glycerol accumulation and seems to be dependent on the Sln1 branch of the pathway (Mollapour and Piper 2006). Fps1 exhibits Hog1 dependent phosphorylation in response to acetic acid, resulting in Fps1 becoming ubiquitinated, endocytosed and degraded, which plays a crucial role in down regulating the acetic acid influx to the cell. Consistent with this data, other mutations that abolish endocytic removal of Fps1 from the plasma membrane, such as loss of Hog1 or mutations generating a general loss of endocytosis of cell surface proteins increased the cell's sensitivity to acetic acid. Fps1 destabilisation does not occur when Hog1 is activated upon hyperosmotic stress. Interestingly, addition of NaCl prior to acetic acid treatment also prevented Fps1 degradation, suggesting that perhaps only the "open" state of Fps1 is destabilised by active Hog1 (Mollapour and Piper 2007).

6. Genetic perturbation on the gene expression level and robustness

Studying how cells or organisms are influenced by gene perturbation is a traditional, established strategy in biological research (Forsburg 2001). Comprehensive gene knock out studies (Giaever et al. 2002) as well as titratable promoter analyses (Mnaimneh et al. 2004) have provided phenotypic information that helps reveal the functions of target genes. In addition, promoter swapping experiments, where the target gene's promoter is replaced by a strong promoter that increases the expression level of the gene of interest, has also made a significant contribution in understanding the impact of gene overexpression. The *GAL1* promoter is a commonly used inducible promoter in *S. cerevisiae*. The *GAL1* promoter can induce strong gene expression in the presence of galactose and is a potent tool for overexpression studies (Sopko et al. 2006). Despite the fact that robustness is an intrinsic feature of life and a fundamental characteristic of biological systems (Kitano 2004), and cells constantly encounter fluctuations and naturally occurring perturbations that lead to dynamic changes of internal parameters, very little is known about the degree to which parameters such as gene expression can be perturbed without disrupting essential functions. We approached this issue by performing a system-wide robustness study on the HOG pathway of *S. cerevisiae*, which is one of the most comprehensively studied eukaryotic signal-transduction cascades (Paper IV).

We applied a genetic screening method known as "genetic Tug- of- War" (gToW) (Moriya et al. 2006), which is an overexpression method. The gToW is based on a 2-micron plasmid vector containing the *leu2-d* allele, a defective allele of a leucine biosynthesis gene *LEU2* (Schneider and Guarente 1991) . In addition, the plasmid carries the gene of interest inserted together with its native regulatory DNA elements (promoter and terminator) as a unit, which is presumed to be natively regulated. The gene expression level is expected to increase according to the copy number of the plasmid. When the *leu2 ura3* mutant yeast transformed with gToW plasmids is cultured under leucine-limiting conditions, there is a bias towards increasing the plasmid copy number to satisfy the requirement for leucine. On the other hand, there is an opposing bias toward decreasing the plasmid copy number if the target gene inhibits growth when a certain copy number is exceeded (i.e. upper limit is reached). Eventually, as a result of the "Tug-of-War" between these two selection biases, cells with optimal plasmid copy number will accumulate (Moriya et al. 2006) and the growth effect can be measured precisely using high-resolution micro cultivation (Warringer and Blomberg 2003).

6.1. Robustness in HOG pathway as a model signal transduction cascade (Paper IV)

Robustness is the ability of a system to maintain functionality despite internal and external perturbations. The robustness landscape contains critical information on the regulation of the signalling network. We focused on the HOG pathway, as a model of the signal transduction cascade in yeast in order to qualitatively search for nodes of fragility and robustness. Applying the gToW methodology, we challenged the HOG pathway with systematic perturbations in components expression level under various external conditions. Our results revealed a high prevalence of negative impacts from gene dose perturbations and a substantially higher frequency of fragile nodes within the HOG pathway (Paper IV, Figure 1A) compared to other cellular processes, such as the cell cycle (Moriya et al. 2006). In total, overexpression of 22 out of 29 HOG pathway components caused significant defects in at least one of the three growth variables (growth rate, growth adaptation time and growth efficiency). Growth rate was considered as primary readout due to its strong correlation with plasmid copy number (Moriya et al. 2006). Whereas there was almost no effect on growth efficiency, the highest frequency and strength of effects were observed during adaptation, possibly indicating the subtle balance of signal transmission in initiating proliferation. It was proposed earlier that overexpression defects are enriched among components that transduce adaptation signals, i.e. kinases, phosphatases and transcription factors (Sopko et al. 2006). Thus, the high occurrence of fragility nodes within the HOG pathway (76%) compared to the cell cycle system (25-30%) (Moriya et al. 2006) might be partly explained by the inherent nature of signal transduction pathways. However, this cannot be the only reason for such a high frequency as the system is also sensitive to overexpression of several components that do not participate in (de) phosphorylation or transcription. We also observed that sensitive targets were dispersed throughout the pathway, independent of biochemical function or location in the pathway topology.

In the next step, we compared our results with the results of a *GAL* overexpression screen conducted on the same targets. Consistent with observations on a cell cycle set (Moriya et al. 2006), an almost complete lack of correlation in terms of cellular toxicity was observed between gToW- and galactose-driven overexpression (Paper IV, Figure S3). In a p*GAL1*-driven overexpression, the gene product is overexpressed without being controlled by its natural promoter, whereas in the gToW method the gene of interest is under control of the native promoter and is expected to be regulated accordingly. Therefore, the difference in the results of the two sets of experiments might partly be explained by the native expression level of each target gene. If the native expression level of the target gene is high, expression due to the *GAL1* promoter will not lead to much overexpression, whereas increasing the copy

number using the gToW method causes a significant increase in overexpression. For instance, *PTC2,* which is highly expressed (Ghaemmaghami et al. 2003), displays a sensitive phenotype upon gToW overexpression. In contrast, *SSK2,* which has a low protein expression level (~217 protein molecules/cell) exhibits a sensitive phenotype upon *GAL* overexpression and no growth defect upon gToW overexpression. Another explanation for the observed differences between the two overexpression methods might be differences in regulation of each target gene. If sensitivity is only observed with the *GAL* method, it is likely that it stems exclusively from the toxicity of the gene product, but gene regulatory mechanisms prevent it from showing up in the gToW screen. Conversely, when sensitivity is only detected with the gToW method, the gene product is most likely not toxic. However, gene regulatory mechanisms or effects such as out-titration of transcription factors cause toxicity at the gene level. The third possible explanation concerns the influence of the GST tag on protein function in the *GAL* overexpression experiments. It has been reported that tagged proteins are not favoured for determining the quantitative effects of a target protein because copy numbers in gToW experiments have been shown to be perturbed by the tags (Moriya et al. 2006).

Furthermore, we showed that the nodes of fragility are largely independent of pathway activation by external stimuli. We applied two types of stress: NaCl stress that activates the HOG pathway and superoxide stress that is not supposed to activate the pathway. The results revealed that the overall gToW sensitivity patterns with both types of stress were very similar to that observed during unstressed conditions, indicating that the nodes of fragility occur regardless of pathway activation (Paper IV, Figure3A). However, the strongest toxicities observed upon overexpression of *PBS2* and *SSK1* were confirmed to be a result of pathway hyperactivation. We found that overexpression of both *PBS2* and *SSK1* activated Hog1 constitutively (Paper IV, Figure 1E) and could be suppressed by the deletion of *HOG1,* indicating that most, if not all, of the toxicity stems from downstream pathway hyperactivation (Paper IV, Figure 1D). In contrast to Ssk1 and Pbs2, the phenotype caused by overexpression of the phosphatase Ptc2 was not mediated through the activation of the HOG pathway and could not be suppressed by deletion of *HOG1* (Paper IV, Figure 1D). Therefore, the mechanism behind its toxicity must be outside the HOG pathway. It has been shown that type 2C protein phosphatases (Ptc2 and Ptc3) are responsible for dephosphorylation of Cdc28, the major budding yeast cyclin dependent kinase (CDK) (Cheng et al. 1999). The fact that CDC28 phosphorylation has been shown to be required for cell cycle progression (Jaspersen et al. 2004) suggests that an increase in the levels of Ptc2 could potentially lower the level of phosphorylated Cdc28, thereby slowing down the cell cycle.

Finally to gain a better understanding of the mechanisms of toxicity of the two most fragile nodes, Pbs2 and Ssk1, we used the existing HOG model (Klipp et al. 2005) to compare the *in*

vivo sensitivity profiles with *in silico* sensitivities concerning dual phosphorylated nuclear Hog1 (Paper IV, Figure2A). The model could neither distinguish the differences in fragility between Ssk1 and Ssk2 nor capture the fragility caused upon *PBS2* overexpression. Therefore, we examined seven variants of the model, which clearly showed that the model structure has a strong impact on the fragility of different nodes. Our results suggest that stable Ssk1–Ssk2 dimer formation (Paper IV, Figures 2D and 2E) and Pbs2 scaffold function (Paper IV, Figures 2F and 2G) contribute to the fragility of their respective nodes.

Taken together, we can conclude that expression changes have a significant negative impact on signalling. The system robustness and fragility is heavily dependent on target components. The observed fragilities are dispersed over the pathway components with no discernible pattern in terms of biochemical function or location of the gene in pathway topology. Furthermore, *in silico* analysis of the studied variants suggests a strong impact of the model structure on the fragility of different nodes.

7. Concluding remarks and future perspectives

I conclude by briefly recapitulating the main points of the thesis, considering future directions.

The N- and C-terminal regulatory domains play an important role in Fps1 regulation (Tamas et al. 2003; Hedfalk et al. 2004). Phosphorylation of the N-terminal regulatory domain inhibits flux through Fps1 both upon arsenite stress as well as acetic acid exposure (Paper I and (Mollapour and Piper 2007). It appears that Hog1 phosphorylation leads to different effects on Fps1 depending on the type of stress condition. Therefore, it would be fascinating to address how different states of Hog1 activation can discriminate between different stress conditions, leading to different outputs in terms of Hog1- mediated Fps1 regulation.

We provide evidence that the MAPK Slt2 contributes to arsenite tolerance by stimulating arsenite efflux through Fps1. We identify a residue in the C-terminal domain that plays role in Fps1- mediated efflux. Direct phosphorylation of Fps1 by Slt2 appears to be the most straightforward explanation, where Slt2-mediated phosphorylation stimulates efflux through Fps1. Whether Slt2 directly interacts with Fps1 and phosphorylates this residue is under investigation.

The *slt2*Δ mutant shows sensitivity in conditions that lead to higher glycerol accumulation. For instance, the *slt2*Δ mutant is sensitive to high temperature (Kamada et al. 1995), in which glycerol is accumulated. Slt2 is transiently phosphorylated following hyperosmotic shock during later phases of the adaptation. This phosphorylation is related to glycerol turn over (Garcia-Rodriguez et al. 2005). It is well known that the *slt2*Δ mutant is sensitive to different types of cell wall stress; we speculate that these phenotypes are partly due to impaired Fps1 mediated glycerol efflux. It will be interesting to further investigate the role of Slt2 in controlling Fps1 efflux activity under conditions that require Fps1 opening.

Regulation of Fps1 by phosphorylation is observed under basal condition as well as arsenite and acetic acid stress. We expect that phosphorylation also plays a role under heat and anaerobic conditions, where a more persistent, fine- tuning response is required. Mechanosensitive gating is more relevant under hyper- or hypo- osmotic stress, where a rapid inactivation or activation of Fps1 is required for survival.

We provide evidence that although the N- and C- terminal domains of Fps1 play a critical role in its regulation, the transmembrane core of the protein plays an equally important role. We suggest a mechanism, in which phosphorylation might influence helix positioning and tilting, thereby affecting the pore of Fps1 in a manner that enables fine-tune of its transport capacity. This mechanism is speculative, addressing how the termini and the transmembrane

core of Fps1 interplay to fine-tune the transport activity of the protein and requires future work.

As phosphorylation appears to be critical in modulating Fps1 transport activity, identification and analysis of additional Fps1 phosphorylation sites might shed new light on understating the modes of Fps1 regulation. So far, 22 putative phosphorylation sites have been suggested (Ammerer et al., unpublished data) however, these data is not comprehensive yet. Applying Phos-tag SDS-PAGE system (Kinoshita et al. 2006), we have succeeded to confirm three Fps1 mobility patterns, two of which were indeed due to phosphorylation (Ahmadpour et al., unpublished data). Nevertheless, much work needs to be done to elucidate the complete phosphorylation pattern of Fps1 and the possible roles of different phosphorylation events.

Identification of the responsible phosphatase(s) is also required for a complete understanding of the molecular mechanism regulating Fps1. In our screen of all protein phosphatase deletion mutants, we found that deletion of a number of phosphatases confer arsenite resistance (Ahmadpour et al., unpublished data). Of particular interest are the *ptp2⊿*, *ptp3⊿* and *ptc1⊿* mutants, which might be explained by their regulatory effect on both Hog1 and Slt2. Interestingly, Ptc1 shows genetic interaction with both Slt2 (Huang and Symington 1995) and Fps1 (Costanzo et al. 2010). Moreover, the different mobility pattern of Fps1 in the *ptc1⊿* mutant supports the possibility that Ptc1 may possibly act on Fps1 (Ahmadpour et al., unpublished data). More work is needed to elucidate the phosphatase(s) involved in Fps1 regulation.

Studying Fps1 interaction partners is another interesting topic that can be further explored. Applying "split-ubiquitin membrane-based yeast two-hybrid system" (Thaminy et al. 2004), two possible physical interactors of Fps1 have been identified. It has been shown that Zeo1, a regulator of the cell wall integrity pathway (Malgorzata Zoltowska et al., unpublished data), and Mdg1, a plasma membrane protein involved in G-protein mediated pheromone signalling pathway (Geijer et al., unpublished data) physically interact with Fps1. The mechanism, by which these interactors affect Fps1 activity, needs to be investigated.

At the systems level, we focused on robustness of HOG pathway against increase in the level of system components. We report that expression changes have a very high impact on signalling, where the observed fragility is largely independent of pathway activation by external stimuli. To expand and deepen the knowledge on the robustness of the entire signalling network, in particular robustness against overexpression of the components is our ultimate goal. We also try to map the interconnectivity of the signalling network by tracing the toxicity effect of fragile nodes. The information on robustness and interconnectivity will eventually be used to re-evaluate and improve the existing mathematical models and will provide a more complete understanding of the dynamic control of the signalling network. Thus far, we have focused on mapping the robustness of TOR, PKA, PKC and PHD/MAT pathways (Walterman et al., manuscript in preparation). The project will be extended to cover

further players in signal transduction i.e. kinases, phosphatases and transcription factors, for which gToW constructs have been constructed. Currently, the transcription factors are verified, phenotyped under normal condition and are being analysed for suppression of fragile nodes (Ahmadpour et al., unpublished data).

Understanding the mechanisms conferring robustness and homeostasis, focusing on water and osmohomeostasis has been the main theme of my thesis. In yeast *Saccharomyces cerevisiae*, an integral membrane aquaglyceroporin Fps1 and a mitogen activated protein kinase Hog1 play essential roles in osmo- and water homeostasis. The studies presented in this thesis, greatly contribute to understanding Fps1 transport activity and regulation and provide new insight into HOG pathway robustness. However, many exciting details remain to be elucidated.

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