Doctoral thesis

From cell populations to single cells

Quantitative analysis of osmotic regulation in yeast

Elżbieta Petelenz-Kurdziel

UNIVERSITY OF GOTHENBURG

Department of Cell and Molecular Biology
University of Gothenburg
Göteborg, Sweden
2010

Mojej Rodzinie

To my Family

"We are not students of some subject matter, but students of problems. And problems may cut right across the borders of any subject matter or discipline."
Karl Popper

From cell populations to single cells
Quantitative analysis of osmotic regulation in yeast

Elżbieta Petelenz-Kurdziel
Department of Cell and Molecular Biology, University of Gothenburg

Abstract

To date, interdisciplinary research is becoming increasingly popular because it combines the achievements of diverse disciplines, having the potential of providing a completely new angle to pertinent research problems. Using increasingly sophisticated tools allowed obtaining large sets of high resolution data but also created the challenge of using this information effectively and interpreting it in a reliable way. Searching for "simplicity in complexity" inspired by engineering and computer sciences, is a new trend in biological sciences, which allows integrating the vast amount of existing knowledge.

Single cell analysis is a good example of interdisciplinary research: dissecting a cell population to specific individuals is at instances necessary in order to obtain information on heterogeneity and cellular dynamics, which might be obscured when investigating, for instance, protein levels in extracts obtained from cell populations. In this thesis I have presented quantitative and time resolved measurements of cellular and nuclear volume, as well as protein shuttling, enabled by the development of a microscope platform dedicated to this type of measurements. I have investigated the response characteristics of the High Osmolarity Glycerol (HOG) pathway in *Saccharomyces cerevisiae* as an example of a MAP kinase network, such as the time scale and amplitude of nuclear Hog1 accumulation, correlated with biophysical changes.

I have also performed experiments on cell populations, aimed at the quantitative characterisation of the downstream effects of the HOG pathway activity, namely glycerol accumulation. In combination of mathematical modelling employing time varying response coefficients, this information allowed us to characterise the importance of each glycerol accumulation mechanism, on different time scales.

In summary, in this thesis I investigated the quantitative aspects of yeast osmotic regulation, providing precise, time resolved information about the biophysical characteristics of osmotic regulation. This work also provides new insight into the network properties of the HOG pathway, indicating the limitations of the response linearity range and the quantitative characterisation of the consequences of HOG activity, namely the interdependence of glycerol accumulation mechanisms. While achieving these goals, I contributed to the development of the single cell analysis platform, dedicated to analysing sub-cellular protein shuttling, correlated with measurements of cellular and nuclear volume.

Contents

Appended Papers

PAPER I
Biophysical properties of *Saccharomyces cerevisiae* and their relation to HOG pathway activation
European Biophysics Journal DOI: 10.1007/s00249-010-0612-0
Authors:
Jörg Schaber, Miquel Àngel Adrover, Emma Eriksson, Serge Pelet, Elzbieta Petelenz-Kurdziel**,** Dagmara Klein, Francesc Posas, Mattias Goksör, Mathias Peter, Stefan Hohmann, Edda Klipp

PAPER II
Quantification of yeast cell volume changes upon hyper-osmotic stress
Manuscript for Integrative Biology
Authors:
Elzbieta Petelenz-Kurdziel, Emma Eriksson, Maria Smedh, Caroline Beck, Stefan Hohmann, Mattias Goksör

PAPER III
Linearity range of the hyperosmotic stress response in *Saccharomyces cerevisiae*
Manuscript
Authors: Elzbieta Petelenz-Kurdziel, Caroline Beck, Roja Babazadeh, Maria Smedh, Emma Eriksson, Mattias Goksör, Stefan Hohmann

PAPER IV
Transcriptional initiation in hyperosmotically regulated genes depends on the osmotic volume recovery rate
Manuscript
Authors: Dagmara Medrala-Klein, Cecilia Geijer, Elzbieta Petelenz-Kurdziel, Abraham Ericsson, Maria Smedh, Marcus Krantz, Mattias Goksör, Bodil Nordlander, Stefan Hohmann

PAPER V
Exploring the impact of osmoadaptation on glycolysis using time-varying response-coefficients
Genome Informatics 2008, 20: 77-90
Authors:
Clemens Kuhn, Elzbieta Petelenz, Bodil Nordlander, Jorg Schaber, Stefan Hohmann, Edda Klipp

PAPER VI
Mechanisms of glycerol accumulation under hyper-osmotic stress and their link to glycolysis
Manuscript for Molecular Systems Biology
Authors:
Elzbieta Petelenz-Kurdziel, Clemens Kuehn, Bodil Nordlander, Dagmara Klein, Kuk-Ki Hong, Therese Jacobson, Peter Dahl, Joerg Schaber, Jens Nielsen, Stefan Hohmann, Edda Klipp

1. Introduction

Integrating diverse fields of knowledge most likely took place since the beginning of scientific research as such, and initially was not given much attention because it was obvious. At the early stage, when different disciplines were not separated, because many of them were just emerging, the paradigm of performing science was slightly different than later on, when very detailed information on specific topics became available. For example, Pascal was conducting research not only on liquid pressure but also on probability and many other topics, whereas the main work of Whatson and Crick was dedicated specifically to discovering the structure of DNA. But when analysing the situation more carefully, also the discovery of Whatson and Crick would not have been possible without the integration of biological knowledge with physics, mathematics and chemistry. In fact, scientific research is and always was interdisciplinary; the possible difference lies rather in the importance assigned to this fact.

Nowadays, interdisciplinary research is more highlighted than it was in the past. The increasing popularity of integrating not only concepts and expertise, but also information and techniques from diverse, also seemingly unrelated research fields, is becoming a more general focus of attention. Researchers seek for collaborations across disciplines, funding authorities are more willing to support projects integrating diverse topics, young people chose study programmes between biology and physics, computer science and linguistics, or mathematics and economy. This phenomenon might partly be a matter of fashion, which influences all aspects of life, including scientific research. But more importantly, interdisciplinary research is becoming so popular because it can be very effective and rewarding.

Combining the achievements of several disciplines allows looking at research problems from a new angle, searching for common patters and possibly finding new interpretations to known facts. Using the benefits of different perspectives and expertise can propel progress immensely and provide new insights, also to matters which seemed to be well understood, but also give rise to even new and interesting questions. A prerequisite for effective collaborations across disciplines is good communication, information flow and constant learning.

The work presented in this thesis is an example of interdisciplinary research, a journey from cell populations to single cell analysis. It integrates the knowledge from biology, physics and mathematical modelling, using the HOG pathway in *Saccharomyces cerevisiae* as an example of a tightly regulated biological process. The work was aimed at quantitative characterisation of HOG response characteristics, combined with the development of tools enabling time resolved single cell analysis of sub-cellular events.

2. Systems biology

Systems biology, although not entirely new as an approach to biological research, constitutes a paradigm shift in the life sciences. It represents a transition from the reductionist approach, which governed biological and medical sciences for several decades, to studying the properties of living entities as networks consisting of functional modules (Ehrenberg, Elf et al. 2009). As a discipline, systems biology develops from the integration of many diverse fields, such as physiology, molecular biology, biochemistry, bioinformatics but also mathematics, computer sciences and control theory. This holistic approach attempts to investigate the dynamic interactions between different components of living systems, therefore shifting the primary importance from examining the role of individual entities, e.g. genes or proteins, to discovering how function arises from their dynamic interactions (Bruggeman and Westerhoff 2007). Yet, the emergence of the systems biology approach would not have been possible without the development of the previously mentioned disciplines. Thus, the systemic approach is a natural continuation of the existing knowledge generating life sciences, propelled by the technical advancements in other disciplines.

The concept of a system as such can be broadly defined as an organised entity, characterised by a defined input and output and a set of features which differentiate it from its surrounding. This entity can be physical or abstract, thus the notion of a system is wide and somewhat arbitrary. The elements of a system are functionally interconnected but this connection is not necessarily reflected by the physical structure of the components. Properties of a system as a whole, along with properties of its individual components, act together to perform the overall function, meaning that neither the component properties alone, nor the system properties alone can explain behaviour of the entire system (Kitano 2002). Properties characterising a system include its structure, dynamics, control method and design principles (Kitano 2002b).

The systemic approach in biology is a search for common patterns of behaviour (Alon 2007). A classic concept presented by Hartwell and colleagues explains the modular structure of biological systems, comparing them to functional modules in electronic devices (Hartwell, Hopfield et al. 1999). Modules are defined as semi-autonomous units performing distinct functions; in biological systems modularity is hierarchical. Following this logic, biological systems can also be viewed as networks, with components represented as nodes and their interactions denoted by edges. Network topology, defined as the interaction pattern between the components, independent of the interaction strengths, is one of the constraints to the overall network functioning; the other constraint is reaction stoichiometry and reversibility (Stelling 2004).

Explaining the behaviour of biological networks from a systems perspective is commonly performed by taking two alternative approaches: “bottom-up” and “top-down”. The “top-down” approach bases on knowledge discovery; it consists in searching for patterns in large datasets, therefore is also called data-mining or the data-driven approach. The other option, called “bottom-up” is referred to as the hypothesis-driven approach. It is based on *in silico* experiments, which are then tested *in vitro* and/or *in vivo* (Kitano 2002; Ehrenberg, Elf et al. 2009). A third approach, which emerged somewhat later is called “middle-out” and is based on a the currently available level of information and then spreads to other levels (Walker and Southgate 2009).

An essential concept in systems biology is the notion of robustness. Robustness can be defined as the ability of a system to maintain its state and functions, in spite of internal and external perturbations. A robust system is relatively insensitive to changes in its internal parameters, but at the same time it can adapt to a changing environment. These seemingly contradictory tasks are performed due to several features such as feedback, modularity, redundancy and structural stability (Kitano 2002b). In reality, there is always a trade-off between robustness and maintenance: very robust systems consume much energy and resources, which at times might not be available. Therefore excessive robustness is not necessarily beneficial. Moreover, a moderate degree of robustness has advantages: very robust systems are less flexible and thus have more difficulties with adjusting to fluctuations of the environment. Therefore, the actual properties of a given system are a compromise between robustness and fragility, meaning the resistance to perturbations and the ability to adapt. As mentioned above, excessive robustness is not necessarily beneficial for living entities: cancer is an example of a very robust system (Kitano 2002).

One of the features, which allow increasing the robustness of a system, is the existence of distinct, semi-autonomous units performing specific functions, so-called modules (Stelling 2004). Each functional module is a discrete entity, which performs a specific function, distinct and autonomous from the functions of other modules (Hartwell, Hopfield et al. 1999). Another system property increasing its robustness is the existence of several autonomous units performing the same function (Kitano 2002), namely redundancy. For example, two input branches of a signalling pathway can be termed as "functionally redundant". This expression does not necessarily mean that one of the branches is dispensable, only that the branches perform equivalent functions. A third feature mentioned in the previous paragraph as a crucial element of robustness is the existence of feedback (Kitano 2002; Stelling 2004). This means of communication between different functional parts of a system conveys output information to the upstream components. The author of a dedicated review defines feedback as "the ability of a system to adjust its output in response to self monitoring" (Freeman 2000). Negative feedback exerts inhibition of the upstream component by the downstream product. Positive feedback does the opposite, boosting the upstream response by the increasing amount of product. Thus, negative feedback increases the stability of a system, contributing to homeostasis, whereas positive feedback increases its variability, also to the extent of switch-like behaviour, which can be advantageous for adaptation (Becksei and Serrano 2000).

Biological networks, in spite of their great diversity, seem to be constructed from a limited set of functional patterns, namely network motives. These basic "building blocks" may help to define universal patterns of networks, since certain network types seem to share particular network motives. For example genetic networks consist of similar motives, which are different from those occurring in ecological food chains or in the World Wide Web. Motives shared by networks of a given type are called "consensus motives" (Milo, Shen-Orr et al. 2002). These motives can be interpreted as a result of the constraints, under which a given network has evolved, and more specifically motifs can be seen as elementary computational circuits performing specific functions (Milo, Shen-Orr et al. 2002). There are specific types of network motifs which exhibit a property called memory. Memory can be defined as a delayed response to a transient stimulus (Ajo-Franklin, Drubin et al. 2007). Motives which exhibit this type of behaviour include mutual inhibition and autoregulatory positive feedback (Ajo-Franklin, Drubin et al. 2007). In the latter case the feedback loop locks irreversibly at a steady state, in response to a transient signal, providing a memory even after the input signal is gone (Alon 2007).

Therefore, it is not surprising that the history of a population of cells influences its behaviour. Other factors, which make a given cell population unique include genetic differences and environmental factors. However, even in a genetically homogenous population differences between cells exist. These differences, termed cell-to-cell variation, arise from random fluctuations of different types, collectively described as biological noise. The noise can originate from gene expression (Raser and O'Shea 2004) – intrinsic noise – but also from fluctuations in cellular components – extrinsic noise (Elowitz, Levine et al. 2002). A comprehensive review on noise and stochasticity in gene expression as a source of cell-to-cell variation was presented by Maheshri and O'Shea (Maheshri and O'Shea 2007).

In order to structure the enormous amount of information in an effective manner, the international scientific community has created a set of standardised conventions for storing and using systems biology data. The Systems Biology Markup Language (SBML) was established as an international standard for mathematical models of biological processes. There are a large number of model repositories, such as:
Kyoto Encyclopedia of Genes and Genomes (KEGG) http://www.genome.jp/kegg/
Alliance for Cellular Signaling (AfCS) http://www.afcs.org/
JWS Online Cellular Systems Modelling (JWS) http://jjj.biochem.sun.ac.za/index.html
The Signal Transduction Knowledge Environment (STKE) http://stke.sciencemag.org/
BioModels Database - A Database of Annotated Published Models
http://www.ebi.ac.uk/biomodels-main/
General Repository of Interaction Datasets (BioGRID) http://www.thebiogrid.org/
Semantic Systems Biology http://www.semantic-systems-biology.org/
just to name a few.

The large number of databases containing information on biological pathways illustrates the prominent role of mathematical modelling in performing systems biology. A model can be defined as an abstract representation of objects or processes, which explains their features (Klipp, Liebermeister et al. 2009). In a wider sense, a model is an entity which mimics and thereby explains crucial features of an object of interest. This means that a model is not necessarily a faithful reproduction of a given process or object, but it shows similarities only in selected aspects and by definition is a simplification. In this sense organisms commonly used in biological research, such as bakers' yeast, are very powerful models for understanding the principles of life. Using living model organisms in order to produce accurate mathematical models requires large amounts of high quality data, which is quantitative and time-resolved (Ehrenberg, Elf et al. 2009).

3. Single cell analysis

Traditionally, biological experiments are performed on large populations of cells using methods based on cellular extracts, such as gel electrophoresis and Western blotting, Northern blotting or microarrays, enzymatic assays and many other, in order to determine gene expression, protein or metabolite levels. A vast majority of these techniques is widely established, usually offering standardised protocols and constant advancements. What would then be the reason for searching alternative methods based on single cell analysis? Population-based experimental approaches, even when optimised, robust and quantitative, offer only a view of the average population behaviour. Such averaged measurements can be misleading, e.g., in the case of a bimodal (or other non-normal) distribution of protein levels (Di Carlo and Lee 2006), where both the kinetics of a response and the measured average value could be misinterpreted. Another example of how bulk measurements could be deceiving is a situation where the output signal is measured to be e.g., 60% compared to the response measured for some other stimuli. Using only data from cell populations, it is not possible to distinguish whether this result means that all cells respond in the same way, reaching 60% of the maximum level or if 60% of the cells respond with 100% intensity, whereas the others remain unaffected. The first type of response is gradual, giving a smooth, 'analogue' response curve, whereas the second type is a switch-like reaction reflected by a steep, 'binary' output. The population behaviour can also be a combination of the two (Figure 1). In addition, the cell response might also vary depending on the cell cycle stage and cell age. Thus, in order to distinguish between the different scenarios, complementary experiments on a single-cell level must be performed. Knowing the behaviour of each individual cell and keeping track from which particular cell a certain signal is derived, is the only way to gain a thorough understanding of a biological process. Such an approach has the power of providing a new type of biological data; nonetheless it usually requires sophisticated tools. Establishing instrumentation, which allows obtaining single cell data, is a real challenge but the effects can be impressive once the development has been made successfully.

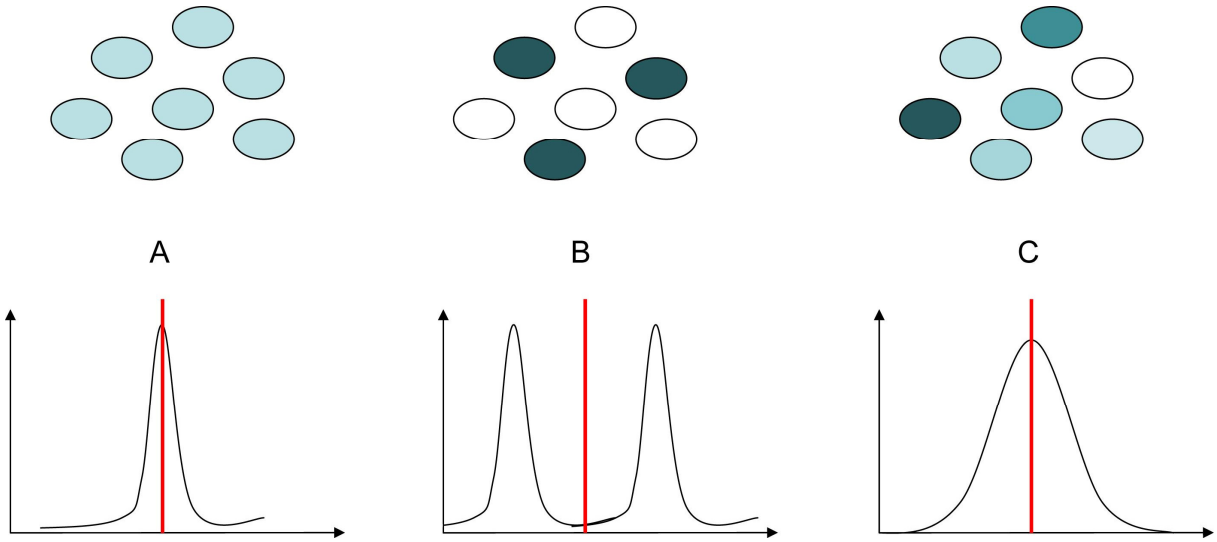

Figure 1: Data extracted from a population of cells represent the average response from all the cells within the population. Thus, it is not possible to distinguish whether the measured response means that all cells respond in the same way (A), in an all-or-nothing fashion (B) or in a combination of the two (C). To understand the mechanisms behind the behaviour of a population, the cells must instead be analysed on an individual basis.

3.1. Dissecting cell populations into fractions

Since calculating a mean from the whole bulk of a cell population consisting of diverse fractions does not necessarily provide useful information, the first step to gain knowledge about such a population of cells is dissecting it into its sub-fractions. One of the means to achieve this is flow cytometry. This method, often referred to as FACS (Fluorescence Activated Cell Sorting), is based on measuring the light-scattering or fluorescent properties of cells, which pass in a narrow stream through a laser beam (Dean and Hoffman 2007). Using this technique, it is possible to obtain high throughput data in a short period of time – ranging from seconds to minutes, depending on the sample density. Cell fractions can be separated based on their properties measured in the flow (Shapiro 2003). However, since the analysed cells are in constant motion, they are lost immediately after the measurement. This excludes the possibility of re-examining a particular cell, once it has been measured. Such a property is an obvious limitation, especially in the combination with the fact that no images are acquired, which potentially hampers data analysis. Therefore, using flow cytometry requires very thorough and carefully planned controls (Alberghina and Porro 1993). In many cases it is necessary to cross-validate the results, using other experimental methods. Another difficulty that can arise while using FACS for yeast is the enormous cell size variation. Since *Saccharomyces cerevisiae* proliferates by budding, the cells are very diverse in size, which results in high standard deviations (Alberghina and Porro 1993).

A technique which attempts to overcome at least some of the above limitations is laser scanning cytometry (Darzynkiewicz, Bedner et al. 1999; Deptala, Bedner et al. 2001). This method allows measuring the fluorescence emitted by labelled cells located on a microscope slide. In contrast to flow cytometry it allows relocating and re-examining particular cells. Moreover several diverse fluorescent dyes can be applied sequentially on a given sample, which can then be stored for additional analysis. An obvious drawback of this method, compared to flow cytometry, is lower throughput and lack of sorting possibilities. On the other hand, laser scanning cytometry cannot provide detailed information about cell morphology, such as images obtained from a microscope. In order to take a further step towards single cell analysis and lab-on-a-chip technology, high content screening (Giuliano, DeBiasio et al. 1997; Pepperkok and Ellenberg 2006; Ye, Qin et al. 2007), where large numbers of cell-containing wells are imaged over time, is a possible option.

It is important to remember that although information obtained on a single cell level has the power to answer many new questions in systems biology, it is still necessary to perform the experiments on a statistically relevant number of cells. Interpreting the behaviour of just a few individual cells as the general behaviour of the entire population may obviously lead to false conclusions (Svahn and van den Berg 2007). Therefore, high quality single cell analysis techniques should provide spatial and temporal information about cellular behaviour, not only with high resolution, but also with relevant statistics. Ideally, such techniques should incorporate precise environmental control and cell manipulation possibilities, since the lack of control of the extracellular micro-environment may contribute to the broadening of population distributions. Possibly, in the future the combination of the single cell technology and large scale studies will bring a new quality to biological research (Pepperkok and Ellenberg 2006), although this kind of development still requires numerous advances, concerning instrumentation, as well as data analysis.

3.2. Methods for single cell analysis

Single cell analysis would not be possible without the parallel advancement of many different technologies: it is usually the combination of several techniques that make this kind of experiments possible. The single cell experiments presented in this thesis rely on the combination of fluorescence microscopy and image analysis, microfluidics and optical trapping.

3.2.1. Fluorescence microscopy

Optical microscopy is an essential tool in biological research. Since its first application by Anthony van Leeuwenhoek in the 17th century, this initially simple device has undergone enormous advancements.

A basic modern microscope consists of the following parts:

1. Illuminator – which includes a light source, collector lens, field diaphragm, and possibly also heat filters, neutral density filters, diffuser.
2. Light conditioner – comprising of the field diaphragm, field lens and condenser.
3. Sample stage – the sample is most commonly located on a glass slide; features which influence the image properties include: slide thickness, cover glass thickness, absorption, transmission and diffraction properties of the specimen, as well as fluorescence emitted by the specimen and by its background.
4. Objective – an essential part of the microscope characterised by its numerical aperture, *NA*:

   $$NA = n \cdot \sin \mu , \qquad (3.2.1.1)$$

   where n denotes the refractive index of the space between the coverslip and the nearest lens of the objective, μ is the half angle of the cone of light which the objective can gather, in other words half of the angular aperture of the objective (Abramowitz 2003). The numerical aperture reflects the light gathering power of the objective: the higher the numerical aperture, the more light a given objective can gather. Geometrical and chromatic aberrations of lenses constituting a microscope objective can be corrected for in various ways, depending on the type and quality of the objective.
5. Eyepiece – which can be characterised by its magnification, field size and eye point.
6. Detector – which can be simply the human eye, but also various devices like photomultipliers, photodiode arrays (CCD), video cameras.

To date there is a huge variety of microscopes in use, with different degrees of complexity, but in all the cases good visualisation of structures depends on two main features: contrast and resolution. Contrast can be defined as the difference in signal intensity between structures of interest and the background, whereas resolution reflects the ability to distinguish two adjacent objects. A concept tightly linked to contrast is the signal-to-noise ratio: the proportion between the intensity of the light emitted by the object of interest and that of its background. It is important to bear in mind that the microscope visualises an image of the specimen, not the specimen itself. This image arises from the diffraction of the rays of illumination light on the details of the imaged object: every point of the object is represented as a set of concentric rings of light around one brighter point – called the Airy pattern – in the focused image plane of the lens. According to Rayleigh's criterion, two point sources are regarded as just resolved

when the principal diffraction maximum of one image coincides with the first minimum of the other (in practice: approximately 200 nm).

At present, a large plethora of contrast enhancing techniques is available, for example: phase contrast microscopy, dark field microscopy or fluorescence microscopy. Fluorescence microscopy, which was extensively used in this thesis, relies on the effect in which a wave of electromagnetic radiation of a given frequency is absorbed by a substance (fluorophore) and an electromagnetic wave of a lower energy (and thus longer wavelength) is emitted by this substance, within nanoseconds. The wavelength difference between the excitation and emission maxima is called the Stokes shift. If the excitation light is filtered effectively from the emitted light, florescence microscopy yields the best contrast available, compared to absorption techniques (Lichtman and Conchello 2005).

When a photon is absorbed by a fluorophore, a transition to an excited state is likely to occur. Thereafter, the excited molecule of the fluorophore gives away the excess of energy by vibrational relaxation and by emitting light, namely fluorescence. The fluorescence emission is characterised by quantum yield (Φ), defined as the ratio between the number of absorbed and emitted photons. The probability of a fluorophore to absorb a photon is described by the molar extinction coefficient (ε) (Lichtman and Conchello 2005). The events which occur in the energy states of a fluorophore can be comprehensively depicted by a Jabłoński diagram (Figure 2).

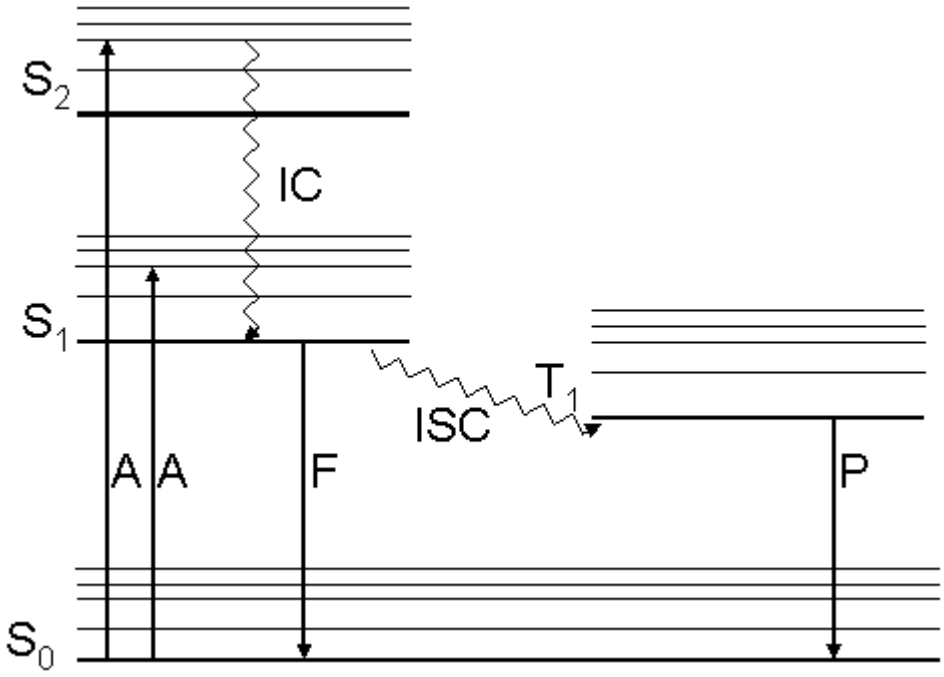

Figure 2: Jabłoński diagram – a schematic representation of luminescence processes. Straight lines – radiative processes, zigzag lines – non-radiative processes. S – singlet states, T – triplet states. A – absorption, F – fluorescence, P – phosphorescence, IC – internal conversion, ISC – intersystem crossing.

Every fluorophore has a characteristic excitation and emission spectrum. The emission is shifted towards longer wavelengths, compared to the excitation. A common problem with the spectral separation, while using several fluorophores is that a given wavelength can possibly excite more than one fluorophore, although to a different extent. As a consequence, the fluorescence emitted by one fluorophore can be detected by the filters used for another fluorophore (Murphy, Piston et al. 2004-2009). This effect is referred to as bleed-through.

Another common problem in fluorescence microscopy is photo-bleaching, i.e. permanent fading of the fluorescent signal, caused by light. Although the exact photochemistry of this phenomenon is still poorly understood, most probably this kind of inactivation is related to triplet states and reactive oxygen species, which react with the fluorophore and irreversibly modify its structure. Theoretically, a good fluorophore should be able to undergo 10 000-40 000 cycles of excitation and emission before it bleaches irreversibly (Lichtman and Conchello 2005). In a great majority of cases, photo-bleaching is an undesired effect in fluorescence imaging, but there are instances, in which it can be used to the advantage. An example is the FRAP (Fluorescence Recovery After Photo-bleaching) technique, where an area of the specimen is bleached deliberately, in order to observe the fluorescence recovery caused by molecule movement.

Another process, which reduces fluorescence intensity is quenching. In contrast to the process of photo-bleaching, a quenched fluorophore fades reversibly. Quenching can be static or dynamic (collisional). In the case of static quenching the fluorophore forms a non-fluorescent complex with the quenching substance, which reduces the overall fluorescence, since the bound molecules cannot be excited. A specific example is so-called self-quenching, where molecules of the same fluorophore bind to each other, preventing excitation (Lichtman and Conchello 2005). Dynamic quenching occurs, when the fluorophore collides with another molecule, usually unexcited and non fluorescent e.g. a halide ion or molecular oxygen. In this case, the overall fluorescence is reduced but this reduction is reversible. An example of such a process is briefly mentioned in PAPER II, where reversible quenching of the dye Calcofluor White by chloride ions was observed. Some sources consider FRET (Förster Resonance Energy Transfer) as an example of dynamic quenching (Lichtman and Conchello 2005). FRET can occur between two molecules, a donor and acceptor, which have a specific spectral overlap: the emission spectrum of the donor matches the excitation spectrum of the acceptor. Another prerequisite for FRET is proximity between the donor and the acceptor molecule. If these conditions are fulfilled, energy from a photon absorbed by the donor can be transferred non-radiatively to the acceptor, which then emits fluorescence. This method can be used e.g. for measuring intermolecular distances, for detecting interactions between molecules or for examining protein folding.

Compounds which are most likely to emit fluorescence have ring structures with conjugated double bonds. Naturally occurring fluorophores (endogenous fluorophores) include aromatic amino acids (phenylalanine, tyrosine and tryptophan), reduced nicotinamide cofactors (e.g. NADH), flavins (e.g. FMN) and porphyrins (Lavis and Raines 2008). These compounds together contribute to what is often called “autofluorescence” – an endogenous background fluorescence of a living cell. Florescence labelling can be performed in various ways, the most simple of which is employing small organic molecules like quinine, fluorescein or rhodamine – just to name a few. These dyes can be used alone or conjugated with antibodies, to stain specific sub-cellular structures. A true revolution in the field of fluorescence microscopy, rewarded with the 2008 Nobel Prize in Chemistry, was the use of genetically encoded fluorescent proteins: GFP (Green Florescent Protein) and its numerous derivatives

(Shaner, Steinbach et al. 2005). Another large step in improving the fluorescence imaging technology was the introduction of quantum dots (Seydel 2003) – semiconductor nano-crystals with very narrow emission peaks. Numerous reviews on florescent labelling describe the advantages and limitations of the different available techniques (Miyawaki, Sawano et al. 2003; Giepmans, Adams et al. 2006).

A wide variety of fluorescence imaging techniques is available to date, but obviously the prevalent approach is epi-fluorescence microscopy. In this setting, the objective is used not only for collecting the light emitted by the sample, but also for illuminating the sample with excitation light. In general, for fluorescence microscopy the objective should preferably have a high magnification and a low numerical aperture. However, in the setup used in this thesis a high numerical aperture is necessary for two reasons. First, 3D optical trapping (see: chapter 3.2.3) requires an objective with a high numerical aperture. Second, fluorescent proteins, which are used as fluorophores, emit relatively weak fluorescence, thus in order to detect it, a high numerical aperture objective is required, to capture as many emitted photons as possible. The light source, which can be an arc lamp, most commonly xenon or mercury (Lichtman and Conchello 2005), or a laser, illuminates the entire field of view with excitation light. A significantly weaker emission light is detected, using sets of filters, both for the excitation and the emission light (Sott , Eriksson et al. 2008), since it is very important that the excitation and emission light are separated effectively (Lichtman and Conchello 2005).

3.2.2. Microfluidics

The term fluidics is used to describe a technique of handling and analysing fluids. In this sense, flow cytometry is also a type of fluidics. The term “micro-fluidics” denotes the use of fluidics on micrometre scale. The main advantage of using microfluidics, apart from the effects of miniaturization, like small liquid volumes and short reaction times, lies in the physics governing micro scale systems (Beebe, Mensing et al. 2002). On such a scale, the principles of device functioning can be very different than those in every day life. A properly designed microfluidic system should use the advantages, which the small scale can offer. Effects, which are at most negligible on macro scale, like laminar flow, diffusion, surface tension or fluidic resistance, become significant in micrometre scale. In order to characterise these effects, a set of parameters is used, i.e. the Reynolds number, diffusion coefficient and fluidic resistance.

The Reynolds number, Re, is given as

$$\mathrm{Re} = \frac{\rho v D_h}{\mu} \qquad (3.2.2.1),$$

where ρ denotes the density of the fluid, v is the characteristic velocity of the fluid, μ is the fluid viscosity and D_h is the hydraulic diameter, which depends on the channel cross-sectional geometry. A Reynolds number below 2300 implies that the flow is laminar, which means that it is possible to predict the position of a particle in the fluid as a function of time. A Reynolds number higher than 2300 indicates a turbulent flow, where the velocity of particles in the stream is random over time, thus calculating their position is not feasible (Beebe, Mensing et al. 2002). In our experiments the Reynolds number is usually below 1.

Diffusion, an effect which is of great importance on micro scale, is as a spontaneous process, during which concentrated particles spread in a volume of liquid, over time, which results in

evening out the concentrations. This effect is driven by Brownian motions. The equation characterising this process is given as:

$$d^2 = 2Dt \qquad (3.2.2.2),$$

where D is the diffusion coefficient, t is time and d is the distance covered by a particle over time. Since the latter value varies to the power of 2, diffusion is effectively much faster on micro scale (Beebe, Mensing et al. 2002).

The liquid velocity inside the channel is given as:

$$Q = \frac{\Delta P}{R} \qquad (3.2.2.3),$$

where Q denotes the flow rate, ΔP is the pressure drop across the channel and R is the fluidic resistance, which depends on the geometry of the channel. The shape of the vessel affects also the relative surface and surface area to volume (SAV) ratio. SAV is important for the diffusion and adsorption parameters, which can affect e.g. the efficiency of pumping. Another important feature is surface tension, caused by the cohesion forces between the liquid particles, on the border between the liquid phase and the gas phase (Beebe, Mensing et al. 2002).

3.2.3. Optical tweezers

The principle of optical trapping lies in the inherent properties of light. Light can be considered either as a wave, or as a stream of particles (the wave-particle duality). A beam of light can undergo refraction or interference but at the same time it consists of myriads of photons. Each photon carries momentum, meaning that it can exert forces on other particles. In the case of light, the conservation of momentum leads at instances to spectacular phenomena, like pushing the tail of a comet away from the sun: the impact of the solar wind forces the gas and dust particles within the comet tail in a direction opposite to its source, resulting in the characteristic shape of the comet. The same effect is used in optical trapping, on a micrometre scale. Radiation pressure, which arises from the light momentum, has the power to trap small, transparent objects. The objects range in size from nanometres to micrometres and can be of very diverse nature – from polystyrene beads, through organelles within the cell to whole cells of bacteria, yeast or other organisms (Ashkin 1997).

Optical tweezers (Ashkin, Dziedzic et al. 1986) consist of a strongly focussed laser beam with a Gaussian intensity profile, meaning that the intensity is stronger in its middle than at the beam peripheries. Thus, light rays differ in their intensities, depending on from which part of the beam they are derived. Two parallel rays of light with different intensities are refracted by the same angle, but the ray with a higher intensity yields with a higher force. From the conservation of momentum, this force must be equilibrated by a counter acting force, which pushes the particle into the centre of the laser beam (Ashkin 1997; Shaevitz 2006). A schematic illustration of this effect is shown in (Figure 3).

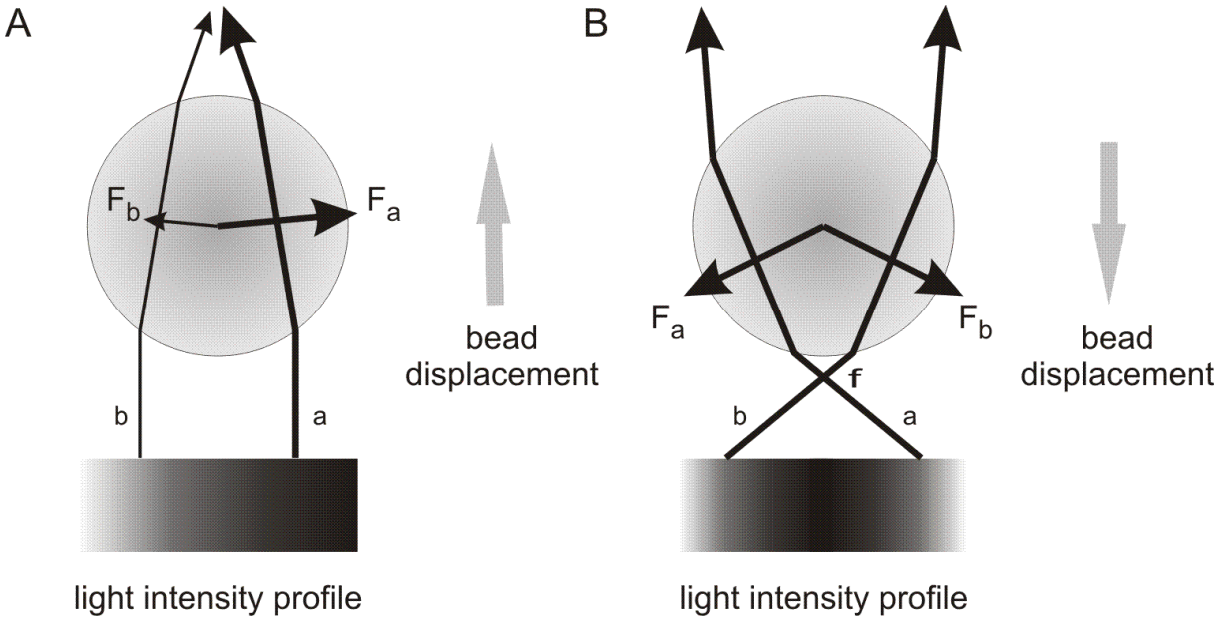

Figure 3: The principles behind optical trapping. A – 2D optical trap. A parallel beam of light interacts with a semi-transparent particle (e.g. polystyrene bead), which has a different refraction index than its surrounding: the ray of light with a higher intensity (a) gives rise to a stronger force (F_a), which compensates the photon momentum change caused by refraction. The bead is pushed towards the centre of the beam and away from the light source (direction indicated by the gray arrow). B – 3D optical trap. A strongly focussed light beam illuminates a semi-transparent bead. Two rays of light with equal intensities (a and b) give rise to equal forces (F_a and F_b), which equilibrate at the focus of the light beam (f). The bead is pushed towards f, where it remains, since at that point the forces are equilibrated. Drawing based on figures in (Ashkin 1997; Shaevitz 2006; Eriksson 2009).

3.2.4. Microfluidics and optical tweezers in biological research

Both microfluidics and optical trapping have many applications in modern biology. Used in combination or separately, they provide refined tools for monitoring biological systems. Micro-channels can be used for sorting, analysing and counting cells, as well as for culturing and monitoring cell physiology (for review see: (Beebe, Mensing et al. 2002)). Optical tweezers, based on an infrared laser beam, were employed for catching and rearranging individual viruses and bacteria, without causing any obvious damage for the analysed objects (Ashkin and Dziedzic 1987). Optical manipulation was also exploited for testing the physical properties of bacterial pili (Jass, Schedin et al. 2004). Another application of optical tweezers is studying motor proteins (for review see: (Ashkin 1997)). A combination of optical tweezers and microfluidic systems was successfully used for monitoring bacterial behaviour in different media (Enger, Goksör et al. 2004), as well as for analysing cell volume changes in yeast, caused by osmotic shock (Eriksson, Enger et al. 2007) and other reactions to a dynamically changing environment (Eriksson, Sott et al. 2010). The setup described in these publications was used to perform experiments for PAPERS I, II, III and IV.

4. HOG as an example of a MAPK pathway

Signal transduction is a process, which allows conveying information from the cell surface to its interior. This task can be achieved by various means. One of them is employing mitogen activated protein kinase (MAPK) pathways. MAP kinase cascades play a crucial role in linking events which occur at the plasma membrane, with cytoplasmic and nuclear responses. MAP kinase pathways share a common architecture (Figure 4), where the core element is the most downstream component: a MAP kinase. The MAP kinase becomes activated due to phosphorylation by its upstream component, a MAP kinase kinase (MAPKK). This phosphorylation occurs on a so-called TXY motif: a tyrosine and a threonine residue, separated by one arbitrary amino acid (Marshall 1994; Cobb and Goldsmith 1995). The MAPKK is activated by phosphorylation, which is mediated by a further upstream component, the MAP kinase kinase kinase (MAPKKK). The MAPKKK in turn is activated, also via phosphorylation, by an external stimulus. This kind of pathway architecture is conserved among basically all eukaryotes, not only from yeast to human (Widmann, Gibson et al. 1999) but also in plants (Caffrey, O'Neill et al. 1999). The external stimuli triggering MAPK cascades are very diverse, like cytokines, growth factors or stress conditions – just to name a few.

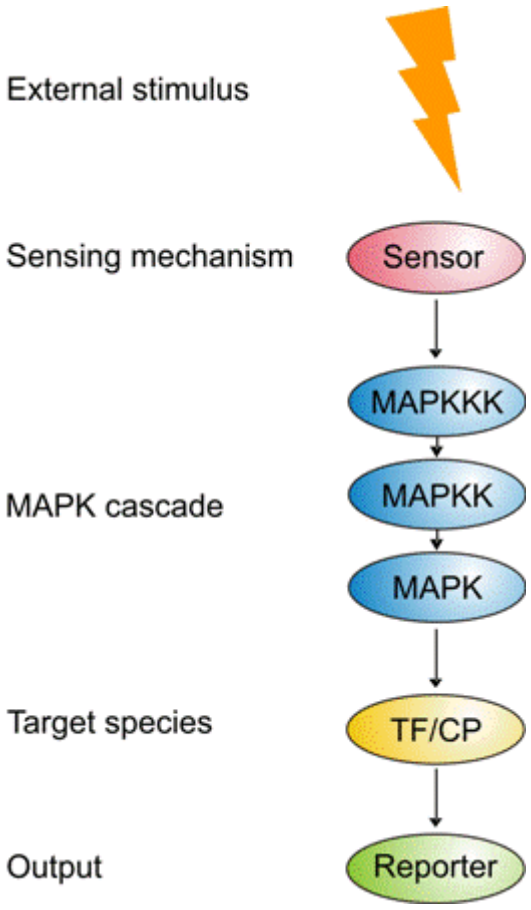

Figure 4: General scheme of a MAP kinase pathway. TF – transcription factors, CP – cytosolic proteins.

MAP kinases have a large variety of targets in the cytoplasm, the nucleus, the cytoskleleton, but also at the plasma membrane itself (Cobb and Goldsmith 1995), as well as many output functions, depending on the context. Therefore, it has been of great interest to determine how pathway specificity is attained. Related to this subject, many other questions arise – about the possible mechanisms of substrate recognition, signalling characteristics like amplitude and frequency, necessary for a given output, interactions between the components of a signalling network, or coupling of the output and input signal (Ammerer 1994; Marshall 1994). Seeking answers to questions concerning control of pathway cross-talk, branching and multiple uses of a signalling machine continuously drives extensive research in the emerging field of systems biology.

The mechanism, due to which MAPK cascades are so common among eukaryotes, is most probably co-duplication (Caffrey, O'Neill et al. 1999). If a gene encoding a signalling pathway component is duplicated, its further fate depends on the presence of other related duplicates. If only a single pathway component undergoes duplication, the functionally redundant gene will eventually be lost by deletion, because its presence very rarely influences the overall fitness. But if two (or even more) genes encoding interacting proteins duplicate together, they have the potential to be maintained, if there presence provides a selective advantage. Such an advantage is more likely if the products of the co-duplicated genes have different specificities than the original ones – then the two pathways can diverge towards different functions. This could be the mechanism driving the evolution of novel pathways, yet the degree of similarity in the functional architecture of MAP kinase networks among very diverse groups of organisms is striking. There are still many questions, which need to be answered. For example, it is a matter of debate whether there existed an ancient orthologous cross-talk, which is now reflected e.g. in the different yeast MAPK pathways sharing components as well as in the mammalian pathways. Alternatively, cross-talk may have evolved independently in different groups of organisms.

An instance, in which genes duplicated and evolved towards different specificities in different organisms are pathways involved in osmotic protection. The mammalian pathways, JNK and p38, most probably arose from the same ancestral hyper-osmolarity pathway as the yeast HOG pathway (Caffrey, O'Neill et al. 1999). Thus, the closest relative of the yeast Hog1 kinase is the mammalian p38, even though it is not the only osmostress-regulated kinase in mammalian cells. The other one, JNK1, is very distantly related to p38 and to Hog1 (Cooper 1994). The mammalian p38 kinase is activated by stress, which can be caused by environmental factors, but also by cytokines mediating inflammation. In its active form, p38 enters the nucleus and phosphorylates serine/threonine residues of numerous substrates. In addition to that, p38 has a prominent role in regulating cell cycle progression, growth, differentiation and apoptosis, as well as in tumour suppression (Dhillon, Hagan et al. 2007). Hence, at cell,level, the physiological roles of Yeast Hog1 and mammalian p38 seem to be very similar.

The High Osmolarity Glycerol response pathway (Brewster, de Valoir et al. 1993) is one of four MAP kinase pathways present in *Saccharomyces cerevisiae* (Hohmann 2009). Since the yeast MAPK network is heavily intertwined, a pathway is defined by a specific input and output as well as the signal transmitting component identified and characterised by genetic and biochemical means. In this sense the input of the HOG-pathway is hyperosmotic osmostress and the output specific adaptive responses mediated by active Hog1. The other three yeast MAPK pathways are:

The pheromone pathway, responding to mating pheromone and mediating the mating response by the Fus3 MAP kinase
The cell wall integrity (PKC) pathway, which responds via the MAPK Slt2 (alias Mpk1) to cell wall stress and controls for cell surface remodelling for instance after hypo-osmotic shock but also after pheromone treatment
The nutrient starvation/invasive growth pathway, which seems to respond to nutrient signals and via the MAPK Kss1 controls morphological changes leading to agar invasion and pseudohyphal growth (Figure 5). Kss1is also involved in the pheromone response (Schwartz and Madhani 2004).
The yeast genome encodes a fifth potential MAPK, Smk1, which is required for spore formation (Gustin, Albertyn et al. 1998; Hohmann 2002; O'Rourke, Herskowitz et al. 2002) however MAPKK and MAPKKK for this pathway have not been identified. If indeed there is such a pathway, the upstream kinases either do not have the characteristic conserved primary structure or the pathway uses MAPKK and MAPKKK from any of the other four pathways.

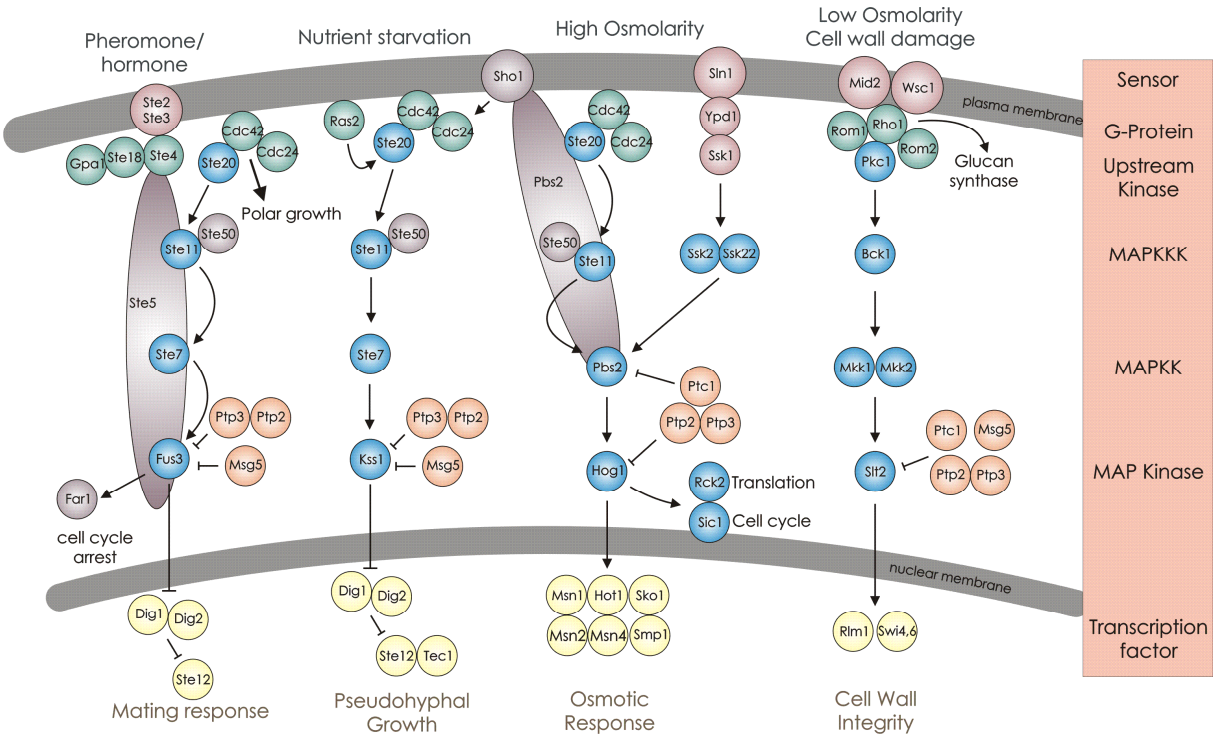

Figure 5: MAPK pathways in *Saccharomyces cerevisiae*.

The HOG pathway consists of two separate input branches, Sho1 and Sln1, named after the two membrane proteins which were discovered first (Maeda, Wurgler-Murphy et al. 1994; Maeda, Takekawa et al. 1995). The Sln1 branch contains a phosphorelay sensing/signalling module similar to bacterial two-component systems, while the initial sensing and signalling of the Sho1 branch is more complex and not well understood. The input branches merge at the stage of the MAPK kinase, Pbs2, resulting in a common output. Active Pbs2 phosphorylates and thereby activates the Hog1 MAP kinase, which enters the nucleus and triggers a large set of transcriptional responses. Hog1 is in fact a transcription factor itself, binding to chromatin via sequence-specific DNA binding proteins such as Hot1 (Alepuz, Jovanovic et al. 2001; Saito and Tatebayashi 2004). In addition, Hog1 has a number of cytosolic targets like the protein biosynthesis machinery (O'Rourke, Herskowitz et al. 2002; Uesono and Toh 2002),

the cell cycle progression apparatus (Escote, Zapater et al. 2004; Clotet, Escote et al. 2006) or the cytoskeleton (Yuzyuk and Amberg 2003). Shutting the activated Hog1 pathway down is a complex event, based on negative feedback (Klipp, Nordlander et al. 2005), but the exact nature of this process is under constant investigation. Deactivation of Hog1 is mediated by phosphatases (e.g. Ptp2, Ptp3) (O'Rourke, Herskowitz et al. 2002; Saito and Tatebayashi 2004), but present understanding suggests that those phosphatase may act constitutively (Klipp, Nordlander et al. 2005). Each part of the HOG pathway is described in more detail in the following sections.

The HOG pathway is not an isolated entity, but part of a larger signalling structure: all the MAPK pathways in yeast can be considered as different modules of the same network. These modules share components like the Ste11 kinase or protein phosphatases. Therefore, mechanisms which allow achieving a particular output, specific to a given input signal while enabling cross-talk and integration of different signals is a field of extensive research (Posas and Saito 1997; O'Rourke and Herskowitz 1998; Schwartz and Madhani 2004; Martin, Flandez et al. 2005). However, such cross-talk is not the main focus of this thesis. This does not mean however, that the wider context is forgotten or that it can be ignored or neglected.

4.1. HOG pathway structure and function

As mentioned previously, the HOG pathway has two separate input branches: Sho1 and Sln1 (Figure 6). The two proteins, from which the branches derive their names, are both located in the plasma membrane but their distribution differs. Sho1 is concentrated in the bud neck and in the newly emerging bud (Reiser, Salah et al. 2000; O'Rourke, Herskowitz et al. 2002), whereas Sln1 is distributed evenly throughout the membrane, possibly excluding the parts occupied by Sho1 (Reiser, Raitt et al. 2003). Moreover, upon hyper-osmotic stress, Sln1 transiently clusters into dot-like structures (Reiser, Raitt et al. 2003). Also structurally these two proteins are not related to each other: Sln1 has an extracellular sensor domain, two trans-membrane domains and two cytoplasmic domains (kinase and receiver), whereas Sho1 consists of four trans-membrane domains and one cytoplasmic SH3 domain (O'Rourke, Herskowitz et al. 2002; Reiser, Raitt et al. 2003).

Why are there two separate HOG inputs? The input branches are functionally redundant in the sense that any of them alone is sufficient for activating Hog1 in response to osmostress (Maeda, Takekawa et al. 1995). The Sln1 branch exhibits a higher sensitivity and gradual reaction characteristics, whereas the Sho1 branch is believed to respond in an 'all-or-none' fashion and to require a higher degree of osmotic stress for activation (Maeda, Takekawa et al. 1995). Some authors consider that those differences may allow achieving accurate responses to a wider range of stress intensities. In that case each of the branches would be specialised in detecting different osmotic conditions (O'Rourke, Herskowitz et al. 2002). Alternatively, the reason might lie in different stimulation mechanisms, i.e. different primary signals perceived by the two sensing devices (Tamas and Hohmann 2003). Also the evolutionary origin of the two branches differs: homologues of the Sln1 branch can be found not only in fungi but also in some other eukaryotes (e.g. *Arabidopsis*, *Dictyostelium* (Stock, Robinson et al. 2000), as well as in numerous prokaryotes, while the Sho1 branch is present exclusively in fungi (O'Rourke, Herskowitz et al. 2002). Interestingly, in most fungal species the Sho1 module has no connection to Pbs2; it is only in *S. cerevisiae* and closely related yeasts where Sho1 is associated with osmotic protection (Furukawa, Hoshi et al. 2005; Krantz, Becit et al. 2006). In conclusion, the reasons for the presence of two input branches in

the HOG pathway are still not entirely clear (Hohmann 2009). More detailed descriptions of each of the branches follows in the next sections.

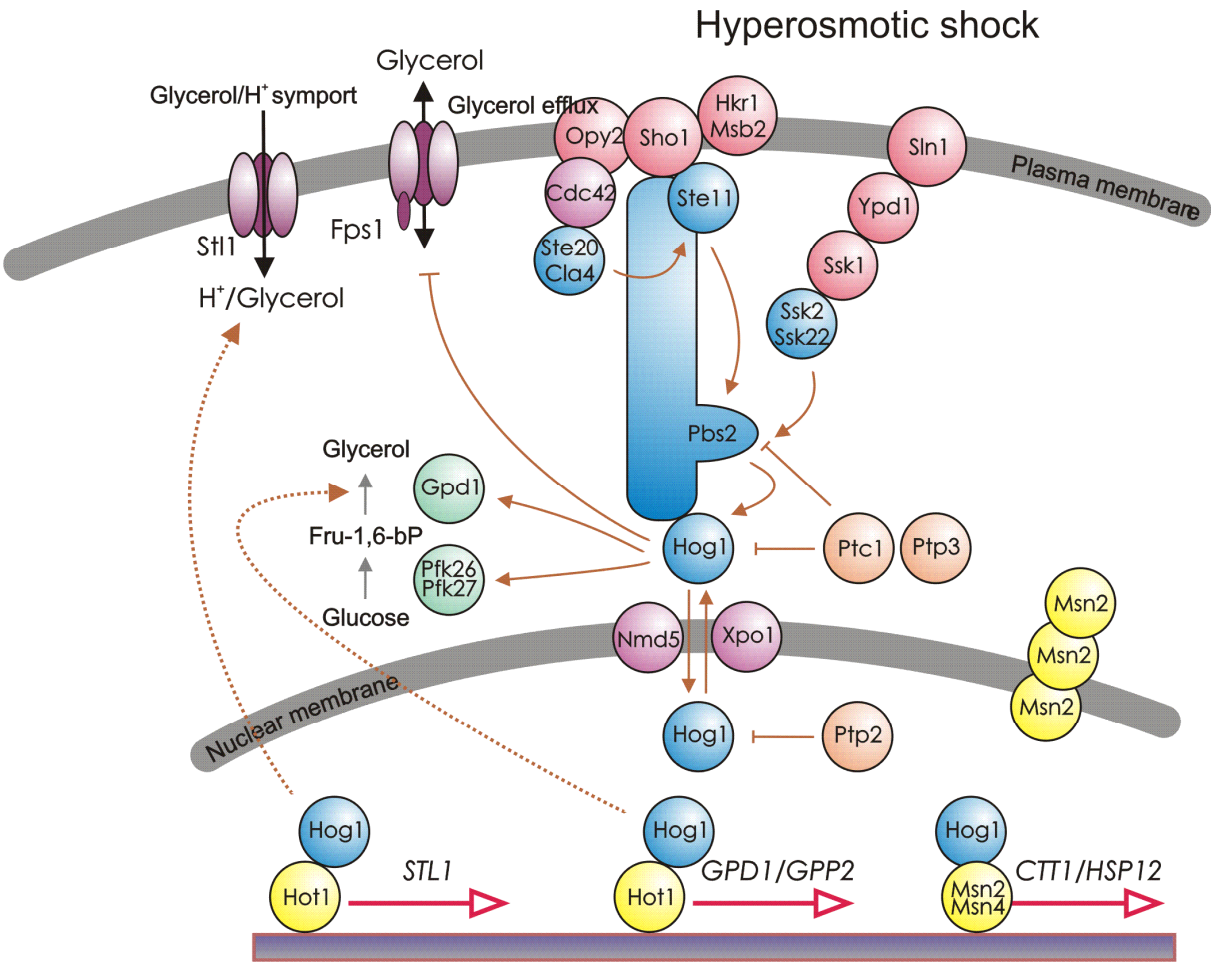

Figure 6: Schematic overview of the HOG pathway. The two branches, Sho1 and Sln1, merge at the stage of the scaffold MAKK, Pbs2. Active Hog1MAPK, imported by Nmd5, enters the nucleus and associates with transcription factors, thereby initiating transcription. Hog1 has also non-nuclear targets, such as Pfk26/27 in the cytosol or the Fps1 aquaglyceroporin in the plasma membrane. Hog1 is exported from the nucleaus by Xpo1.

4.1.1. Sln1 branch

The Sln1 branch is controlled by a phospho-relay module, which is homologous to a two-component system. It consists of three elements: Sln1, Ypd1 and Ssk1.

A two-component system is the simplest transduction mechanism possible. As the name indicates, it consists of just two elements: a sensor and a response regulator. The sensor has an input domain, which is activated by an input signal, and a transmitter module, which conveys the signal to the receiver module of the response regulator. The receiver module of the response regulator activates an output domain. This generates an output signal, which in turn triggers an output response. Most commonly the signal conveyed is a phosphate group, which first binds to a histidine residue in the sensors transmitter module and subsequently is transferred to an aspartate residue in the receiver module of the response regulator (Parkinson

and Kofoid 1992). This type of signalling module is prevalent among prokaryotes, but also occurs in plants and fungi, including *Saccharomyces cerevisiae*, but not in animals (Posas, Wurgler-Murphy et al. 1996; Posas and Saito 1998; Stock, Robinson et al. 2000; West and Stock 2001). The eukaryotic version commonly consists of a series of phospho-transfer events and more than two components, hence the name phosphorelay.

In the case of the Sln1 branch of the HOG pathway, the sensor is Sln1 itself – it appears to respond to changes in turgor pressure (Reiser, Raitt et al. 2003). Sln1 is a histidine kinase, located in the plasma membrane. It contains one extracellular sensor domain and two cytoplasmic domains: a histidine kinase domain and a receiver domain. This is an interesting modification of the classic two-component system, where a receiver domain is present only in the response regulator. Ssk1, which acts as the actual response regulator, contains another receiver domain and is located in the cytoplasm. The third physical component of this "two-component" system is Ypd1, which binds to Sln1 as well as to Ssk1, mediating transfer of a phosphate group. The transfer of the phosphate group begins with autophosphorylation of a histidine residue in Sln1. In fact, it has been shown that phosphorylation occurs in trans between the two subunits of the obligatory Sln1 dimer (O'Rourke, Herskowitz et al. 2002; Hohmann 2009). From the histidine residue of Sln1 the phosphate is passed on to an aspartate group within the same protein, then to a histidine group of Ypd1, and finally to an aspartate group of Ssk1 (Posas, Wurgler-Murphy et al. 1996).

Sln1 remains phosphorylated under normal osmotic conditions, but when external osmolarity increases, Sln1 is dephosphorylated. Therefore, Sln1 cannot convey the inhibiting phosphate group to Ssk1 and unphosphorylated Ssk1 begins to accumulate (Posas, Wurgler-Murphy et al. 1996). Unphosphorylated Ssk1 binds to the regulatory domains of Ssk2 and Ssk22 and releases their autoinhibitory domains, resulting in autophosphorylation (Posas and Saito 1998). Since Ssk2 and Ssk22 act as MAPKKKs in the Sln1 branch, this event triggers the MAP kinase cascade.

4.1.2. Sho1 branch

The branch got its name after Sho1, the first membrane protein linked to the pathway and long believed to be a sensor. However, it now appears to rather serve a scaffold function (Maeda, Takekawa et al. 1995; Tatebayashi, Yamamoto et al. 2006). In the past it was also thought that there is a third activation branch of the HOG pathway (Van Wuytswinkel, Reiser et al. 2000; O'Rourke and Herskowitz 2002), in which the mucin Msb2 plays an important role. Later it became clear that Msb2 is in fact an upstream element of Sho1. Now it is believed that the transmembrane mucins, Hkr1 and Msb2, act as sensors in the Sho1 branch (de Nadal, Real et al. 2007; Tatebayashi, Tanaka et al. 2007), possibly by monitoring the movements of the cell wall in relation to the plasma membrane (O'Rourke and Herskowitz 2002; Hohmann 2009).

Given that Sho1 is located in the plasma membrane, but not involved directly in sensing osmolarity changes (Tamas and Hohmann 2003), its main function is probably that of a scaffold. The localisation of Sho1 at sites of polarised growth, especially in the bud neck (Reiser, Salah et al. 2000; O'Rourke, Herskowitz et al. 2002), is to a large extent independent of the actin cytoskeleton: it is the small Rho-type GTP-ase, Cdc42, which initiates sites of polarised growth by organising the actin cytoskleleton, so that protein complexes can be assembled around it (Reiser, Salah et al. 2000). Active GTP-bound Cdc42 recruits Ste20 to

the plasma membrane, where it is attached, and renders Ste20 active. Ste20 is a PAK-like (p21-activated) kinase, which activates Ste11 by a conformational change that removes the amino-terminal inhibitory domain from the catalytic domain (Drogen, O'Rourke et al. 2000). Cla4 is another PAK-like kinase, executing the same function as Ste20 (Tatebayashi, Yamamoto et al. 2006). The activity of Ste11 is also modulated by Ste50, which is an adaptor protein constitutively bound to it (Tatebayashi, Yamamoto et al. 2006; Wu, Jansen et al. 2006). Ste50 brings Ste11 to the plasma membrane and links it with the Cdc42-Ste20 complex. The complex formed by Ste11 and Ste50 is initially attached to the plasma membrane by Opy2 (Wu, Jansen et al. 2006) but then it is passed on to Cdc42. Cdc42 has an active role in binding the Ste11-Ste50 complex and bringing activated Ste20/Cla4 in the proximity of their substrate Ste11 (Tatebayashi, Yamamoto et al. 2006). Cdc42 is also necessary for localising Pbs2 to the plasma membrane (Reiser, Salah et al. 2000). Sho1 not only binds Ste11 and mediates its activation, but it also brings it together with Pbs2, which it binds via the SH3 domain (Zarrinpar, Bhattacharyya et al. 2004). Thus the role of Sho1 is important for assembling active protein complexes, as well as for anchoring Pbs2 to the cell surface (Posas and Saito 1997). However, it is not only Sho1, but also Cdc42 and Ste50 that control the signal flow from Ste20/Cla4 to Ste11and finally to Pbs2, by acting as adaptor proteins (Tatebayashi, Yamamoto et al. 2006).

Also Pbs2 is an active element of the whole process: it is not only a MAPKK, but also a scaffold protein. A prerequisite for Pbs2 activation is its association with Ste11 (Posas and Saito 1997). Sho1 and Pbs2 act as co-scaffolds, either in a sequential or in a cooperative manner, once active Ste11 binds to them. This cooperation between Sho1 and Pbs2 was compared to the function executed by Ste5, which is a scaffold protein in the pheromone pathway (Zarrinpar, Bhattacharyya et al. 2004). Activation of the MAPKK Pbs2 is the stage, at which the two branches of the HOG pathway merge.

4.1.3. MAPK cascade

The MAPK kinase Pbs2 can be activated by three alternative MAPKKKs: Ssk2 and Ssk22 from the Sln1 branch and Ste11 from the Sho1 branch (Posas and Saito 1997). As mentioned in section 4.1.1., both Ssk2 and Ssk22 undergo autophosphorylation, if their inhibition ceases due to the presence of unphosphorylated Ssk1. Pbs2 has a specific docking site for Ssk2/Ssk22. This docking site most probably increases the signalling efficiency and is not used by Ste11 (Tatebayashi, Takekawa et al. 2003). The same authors postulate that Pbs2 could be constantly bound to Ssk2/Ssk22 in a pre-activation complex, also in the absence of hyper-osmotic stress. Upon stress, the accumulated excess of unphosphorylated Ssk1 would release the autoinhibitory domain of Ssk2/Ssk22, allowing it to achieve an open conformation and to activate Pbs2.

The activation of Pbs2 through the Sho1 branch is even more elaborate. As described in section 4.1.2., in order to become phosphorylated by Ste11, Pbs2 must be attached to the plasma membrane and brought into proximity with the Ste50-Ste11 complex. This membrane attachment of Pbs2 is mediated by Sho1. But at the same time, Sho1 and Pbs2 act as co-scaffolds for binding and activating the MAPKKK, Ste11 (Zarrinpar, Bhattacharyya et al. 2004; Tatebayashi, Yamamoto et al. 2006). Therefore, Pbs2 is not only a MAPKK, but also an active platform for the preceding stages of this multi-step process of signal transduction.

Pbs2 is situated only in the cytoplasm, and not in the nucleus, regardless of osmotic stress (Ferrigno, Posas et al. 1998; Reiser, Ammerer et al. 1999; Reiser, Salah et al. 2000). This localisation is probably caused by a nuclear export (NES) sequence (Tatebayashi, Takekawa et al. 2003). It has been demonstrated that Pbs2 is the only activator of Hog1 (Brewster, de Valoir et al. 1993; O'Rourke and Herskowitz 2004).

Hog1 is a homologue of the mammalian p38 MAPK, both at sequence and functional level (Caffrey, O'Neill et al. 1999). Phosphorylation of Hog1 takes place within less than a minute after hyper-osmotic shock (Reiser, Ammerer et al. 1999; Hohmann 2009). It is a dual phosphorylation, which occurs on threonine 174 and tyrosine 176 –phosphorylation sites conserved among other MAP kinases (Brewster, de Valoir et al. 1993; Choi, Kang et al. 2008). It is not completely clear if the two residues are phosphorylated in a certain order but it appears that both sides must be phosphorylated for Hog1 to be active. In the absence of stress, Hog1 is evenly distributed in the cytoplasm, whereas upon hyper-osmotic shock phosphorylated Hog1 accumulates inside the nucleus (Ferrigno, Posas et al. 1998; Reiser, Ammerer et al. 1999). The nucleo-cytoplasmic shuttling of Hog1 is mediated by the importin Nmd5 and the exportin Xpo1 (Ferrigno, Posas et al. 1998).

4.1.4. Transcriptional response

Hog1, once it enters the nucleus, associates with transcription factors such as Hot1, Smp1, Msn1, Msn2, Msn4 and Sko1 (Rep, Reiser et al. 1999; Rep, Proft et al. 2001; Saito and Tatebayashi 2004; de Nadal and Posas 2010) and participates in initiating transcription. In some instances (Hot1, Msn2 and Sko1; (Rep, Reiser et al. 1999)) is has been shown that Hog1 is recruited to target promoters by these DNA-binding proteins and activates transcription itself. Upon hyper-osmotic conditions, the whole process of transcription, beginning with the assembly of the pre-initiation complex (PIC), followed by the actual transcription initiation, elongation and finally termination, involves various Hog1 actions (Proft, Mas et al. 2006; de Nadal and Posas 2010), a few of which are described below. Possibly additional ones remain to be discovered.

Hog1 associates with *GPD1* chromatin. It has been shown that this association depends on Hot1, and not on increased nuclear transport of Hog1, caused by hyper-osmotic stress (Alepuz, Jovanovic et al. 2001). The same authors demonstrated that Hog1 binding to *CTT1* and *HSP12* does not depend on Hot1, but on Msn2 and Msn4, whereas the binding of Hog1 and Hot1 to *STL1* is interdependent. The latter gene is commonly used a HOG pathway activity reporter.

The functional interaction of Hog1 with Hot1, but also with Msn1, also triggers the expression of *GPP2* (Rep, Reiser et al. 1999). Msn1 is a transcription activator with similarity to Hot1. It is involved, among other processes, in the osmotic stress response, in particular in *CTT1* up-regulation (Rep, Reiser et al. 1999). Msn2 and Msn4 are general stress protective transcription factors. They are functionally redundant zinc finger proteins, necessary for the transcription of several stress-induced genes, including those related to osmotic stress (Martinez-Pastor, Marchler et al. 1996; Alepuz, Jovanovic et al. 2001). Cells lacking all the HOG-related transcriptional activators i.e. Msn1, Msn2, Msn4 and Hot1, fail to up-regulate HOG-dependent and general stress-dependent genes, like *GPD1*, *GPP2*, *CTT1* and *HSP12*, upon hyper-osmotic stress (Rep, Reiser et al. 1999). Interestingly, such cells also show a

decreased level of nuclear Hog1 compared to wild type cells (Rep, Reiser et al. 1999). At the same time such quadruple mutants do not display osmostress sensitivity, suggesting that transcriptional responses are not totally required for osmotic adaptation. This notion was recently confirmed by employing an engineered Hog1 that cannot enter the nucleus (Westfall, Patterson et al. 2008).

The transcriptional repressor Sko1 is phosphorylated in a Hog1-dependent manner, which results in a switch in Sko1 function, from repression to activation. This is mediated by the modification of its association with the Tup1-Ssn6 complex (Rep, Proft et al. 2001; Proft and Struhl 2002; de Nadal and Posas 2010). One of the target genes for the Hog1-Sko1 complex is *GRE2*, which is another commonly used HOG reporter (Rep, Proft et al. 2001).

Besides being critical for transcription initiation Hog1 participates inside the nucleus also in chromatin remodelling (de Nadal and Posas 2010) and interaction with RNA polymerase II during mRNA elongation (Proft, Mas et al. 2006; de Nadal and Posas 2010).

As indicated above, if Hog1 is unable to enter the nucleus (which can be achieved by tethering it to the plasma membrane with farnesyl and palmitoil groups), the cell is still able to adapt to increased external osmolarity, even though all the transcriptional effects are abolished. This indicates that Hog1-mediated transcriptional responses are not essential for survival under hyper-osmotic stress. Probably, those transcriptional responses are long-term adaptations and set a new steady-state of adapted cells rather than being important for the actual adaptation. For this, the metabolic control of glycerol production and transport may be more important, which was rather unexpected.

4.1.5. Shutting the pathway down

MAP kinase pathways play a prominent role in regulating cell division, morphogenesis and physiology. Therefore it is crucial that their activity is tightly controlled because both too low or too high activity, or inappropriate timing of activation, can be detrimental or even lethal. Activating MAP kinases happens by phosphorylation and hence deactivation occurs by dephosphorylation catalysed by phosphatases. In yeast, phosphatases not only restrict MAP kinase activity but also control their sub-cellular localisation and prevent cross-talk between different pathways (Martin, Flandez et al. 2005).

There are three main groups of protein phosphatases: serine/threonine phosphatases, protein tyrosine phosphatases and dual specificity phosphatases (Martin, Flandez et al. 2005). The phosphatases involved in the HOG pathway, namely Ptc1, Ptc2, Ptc3 (Warmka, Hanneman et al. 2001), Ptp2 and Ptp3 (Martin, Flandez et al. 2005), belong to the first two groups. In the absence of hyper-osmotic stress, phosphatases decrease basal pathway activity (Saito and Tatebayashi 2004). Deletion of the genes *PTP2* plus *PTC1* results in constitutive activation of Hog1, which in turn results in lethality (Maeda, Wurgler-Murphy et al. 1994).

Ptp2 and Ptp3 are protein tyrosine phosphatases. Ptp2 is located in the nucleus, Ptp3 – in the cytoplasm. The transcription of *PTP2* and *PTP3* is moderately up-regulated after hyperosmotic shock in a Hog1-dependent manner, which probably is important for setting a new steady-state in osmoadapted cells (Wurgler-Murphy, Maeda et al. 1997). Ptc1, which belongs to the serine/threonine phosphatases, dephosphorylates Hog1 during adaptation after

hyper-osmotic stress and maintains its low basal activity, whereas Ptc2 and Ptc3 limit the maximal level of Hog1 activation, but a pronounced *in vivo* effect can be obtained only when *PTC2* is over-expressed (Young, Mapes et al. 2002). The *ptc2Δptc3Δ* mutant shows Hog1 hyper-activation upon increased external osmolarity. Ptc2 and Ptc3 have a very high degree of similarity but differ to some extent from Ptc1 (Young, Mapes et al. 2002).

4.1.6. HOG network analysis

Several studies dedicated to examining the network properties of the HOG pathway have been published in the last few years. Two independent groups investigated the response of the HOG pathway to periodic stimuli (Hersen, McClean et al. 2008; Mettetal, Muzzey et al. 2008). Hersen and colleagues examined the capacity of the HOG pathway to carry information, addressing the question: how much information can be processed by a given pathway in a unit of time? Using a microfluidic device for applying rapid environmental changes the authors observed that the HOG pathway reacts faithfully to repetitive stimuli of moderate frequencies, but once the frequency reaches a certain threshold the pathway integrates input signals, reacting to their average value. The integration is caused by the accumulation of active pathway components during each on-phase, which happens when the off-phase is too short for the pathway to become completely deactivated. The authors found this threshold frequency to be 4.6×10^{-3} Hz: at frequencies lower than this value, the amount of Hog1-GFP observed inside the nucleus faithfully follows the input (Hersen, McClean et al. 2008). This frequency, referred to as the bandwidth of the pathway response, reflects the amount of information which a given pathway can carry.

Mettetal and co-workers also examined the frequency dependence of osmotic adaptation in *S. cerevisiae*, but with focus on signal propagation properties. Based on a mathematical model, the authors postulate the existence of two feedback mechanisms, one Hog1-dependent, the other Hog1-independent, contributing to the overall adaptation: accumulating glycerol (Mettetal, Muzzey et al. 2008). The authors found that the overall HOG response is dominated by the fast acting negative feedback, which does not require protein synthesis. Only for strong osmotic stresses the transcriptional response becomes important and possibly the gene products prepare the cell for subsequent osmotic shocks, which might occur in the future.

The findings of the two groups are summarised in a commentary, with a very adequate title: *"How fast is too fast?"*, by Paliwal and colleagues (Paliwal, Wang et al. 2008), discussing how design experiments can provide information about the properties and limitations of cellular signal processing.

Dynamic signalling through the HOG pathway was also examined by Macia and co-workers (Macia, Regot et al. 2009), in the context of input thresholds, taking into account the properties of individual input branches. This group observed that the HOG pathway exhibits intrinsic basal signalling, whereas Paper III of this thesis is dedicated to examining the linearity of the HOG response, for very strong input signal. Another example of investigating HOG network properties is the extensive study on robustness and fragility, by Krantz and colleagues (Krantz, Ahmadpour et al. 2009), which shows that the distribution of the fragile nodes is dispersed around the pathway. Although the fragilities do not show any clear pattern

related to their location in the pathway topology, the strongest toxicity of the fragile nodes is a result of their hyper-activation.

4.2. Biophysical changes

The HOG response is initiated by increased external osmolarity; which are the underlying biophysical changes that trigger the HOG pathway?

The term "osmosis" denotes the spontaneous passage of a solvent, in this case water, through a semi-permeable membrane, meaning that the solvent but not the solute can pass through this membrane. The pressure which must be applied in order to prevent the solvent influx through the membrane is called the osmotic pressure (Atkins 1998). Osmotic pressure is given by the van't Hoff equation:

$$\Pi = [B]RT \qquad (4.2.1),$$

where Π is the osmotic pressure, $[B]$ is the molar concentration of the solute, R is the gas constant and T is the absolute temperature. Another important concept which should be introduced is chemical potential. The chemical potential μ of a pure substance is defined as:

$$\mu = \left(\frac{\partial G}{\partial n}\right)_{T,p} \qquad (4.2.2),$$

where G denotes Gibbs energy, n is the number of moles of the substance, T is the absolute temperature and p is the pressure (Atkins 1998). The chemical potential μ_A of a solvent A is given as:

$$\mu_A = \mu_A^* + RT \ln a_A \qquad (4.2.3),$$

where μ^*_A is the chemical potential of pure A, a_A is the activity of solvent A, R is the gas constant and T is the absolute temperature (Atkins 1998). Molarity describes the molar concentration of a substance, while molality indicates the number of moles of a given substance per 1 kg of solute. Respectively, osmolarity is the number of osmoles per litre of solvent, i.e. the number of moles of solute contributing to the osmotic pressure of a given solution, while osmolality refers to 1 kg of solvent. Namely, osmolarity indicates the molar amount of solutes present in the solution. Even though osmoles are not SI units, the concept of osmolarity is widely used due to its convenience. Intuitively, the chemical potential of water is the effective water concentration in a given area. This means that water flows towards the region with a lower water potential (and thus with a higher osmolarity). Therefore, the chemical potential of water is the driving force of osmotic regulation in living organisms. For more details about the definitions please refer to handbooks on physical chemistry, e.g. (Atkins 1998).

Another biophysical characteristic relevant for understanding osmotic regulation is turgor. Turgor is defined as a pressure which compensates the difference of osmotic pressures between the cell interior and the extracellular environment, caused by the stiffness of the cell wall:

$$\Pi_t = \Pi_i - \Pi_e \qquad (4.2.4),$$

where Π_t denotes turgor pressure, Π_i is the osmotic pressure inside the cell and Π_e is the osmotic pressure of the cell surrounding, at equilibrium (Klipp, Nordlander et al. 2005; Gennemark, Nordlander et al. 2006). In other words, turgor is a hydrostatic pressure exerted on the cell wall by the protoplast. Under normal conditions the osmotic pressure inside the cell is slightly higher than that in its surrounding. When the external osmotic pressure rises,

turgor decreases rapidly. Turgor is an important parameter in mathematical models of the HOG pathway (Klipp, Nordlander et al. 2005; Gennemark, Nordlander et al. 2006). Even though to date, there is no method of measuring turgor directly, this value can be derived from cell volume (Schaber and Klipp 2008; Schaber, Adrover et al. 2010) as:

$$\Pi_t = \varepsilon \frac{dV_m}{V_m} \qquad (4.2.5),$$

where Π_t is the turgor pressure, ε is the volumetric elastic modulus (Cosgrove 1988), characterising the stiffness of the cell wall and V_m is the volume enclosed in the plasma membrane. The cellular volume itself consists in fact of several volumes, exhibiting diverse behaviours:

$$V_m = V_{os} + V_b \qquad (4.2.6),$$

where V_m is the membrane enclosed volume, V_{os} is the osmotic volume, i.e. the volume which changes in response to shifts in external osmotic pressure and V_b is the basal solid volume, meaning a size, beyond which the cell cannot shrink, regardless of the osmotic conditions. Because the cell wall is stiffer (Smith, Zhang et al. 2000; Harold 2002) than the plasma membrane, at very high osmolarities it can reach a point when further shrinkage is impossible, whereas the protoplast can continue decreasing in volume. In this situation the plasma membrane detaches from the cell wall in a process termed plasmolysis; this detachment begins at the so called plasmolytic point. Therefore the volume of the cell wall can be described as the apparent volume:

$$V_{ap} = V_m + V_{pl} \qquad (4.2.7),$$

where: V_{ap} is the apparent volume, V_m is the membrane enclosed volume and V_{pl} is the plasmolytic volume, meaning the space between the cell wall and plasma membrane. The above concepts, as well as other aspects related to turgor and cell volume changes are addressed in detail in Papers I (Schaber, Adrover et al. 2010) and II.

4.3. Accumulation of glycerol as a compatible solute

The adaptation to increased external osmolarity, which results in a drop of cell volume and turgor pressure, requires the accumulation of physiologically neutral osmotically active compounds – so-called compatible solutes. The function of a compatible solute is to maintain cell volume during osmotic stress (Yancey 2005) and to protect the enzymes present in the cell from inhibition or inactivation resulting from decreased water activity (Brown 1974). This is possible due to the very weak interactions (Brown 1974) or even stabilising effects (Yancey, Clark et al. 1982) of the compatible solute on the biomolecules present in the cell. Most of all, the accumulation of osmotically active compounds drives water influx into the cell: it is not the absolute osmotic pressure but the pressure difference between the intracellular and extracellular environment, which determines the direction of water flux. Thus, even very mild changes in the intracellular osmolarity can have immense consequences for the cell functioning (Davis, Burlak et al. 2000).

The compatible solute employed by growing cells of baker's yeast is glycerol – a three-carbon poly-alcohol. It is the predominant by-product of ethanol fermentation in *Saccharomyces cerevisiae* (Cronwright, Rohwer et al. 2002) and, at the same time, a compatible osmolyte. Glycerol metabolism also participates in maintaining the redox balance under anaerobic and glucose repressing conditions (Andre, Hemming et al. 1991; Ansell, Granath et al. 1997; Yancey 2005). To date, there are four known mechanisms contributing to glycerol accumulation upon hyper-osmotic stress, in *Saccharomyces cerevisiae*, which all are affected

by the HOG pathway: uptake from the external environment via Stl1, outflow prevention due to Fps1 closure, glycerol production, catalysed by Gpd1 and Gpd2, and stimulation of glycolysis via Pfk26 and Pfk27. A schematic representation of these mechanisms is shown in Figure 7; their interdependence is addressed in Paper V (Kuhn, Petelenz et al. 2008) and Paper VI (Petelenz-Kurdziel, Kuhn et al. 2010).

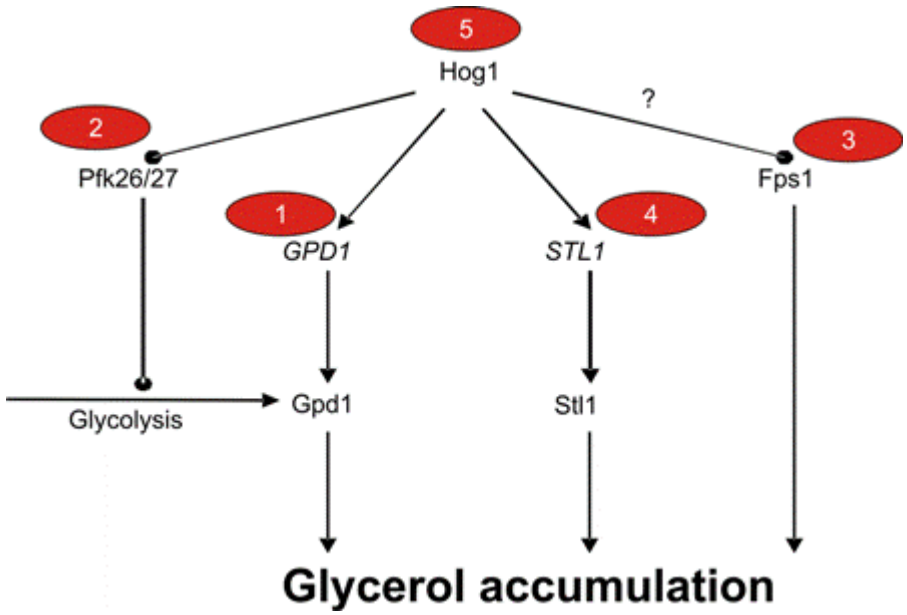

Figure 7: Mechanisms of glycerol accumulation.

4.3.1. Glycerol production

The primary source of glycerol in the cell is production from dihydroxyacetone phosphate (DHAP). The process occurs in two steps: first DHAP is converted to glycerol-3-phosphate (G3P), and then G3P is dephosphorylated to glycerol. The enzyme catalysing the latter step, glycerol-3-phosphatase (Gancedo, Gancedo et al. 1968), exists in two isoforms: Gpp1 and Gpp2 (Norbeck, Påhlman et al. 1996), known also as Rhr2 and Hor2, respectively (Hirayama, Maeda et al. 1995).

But the rate limiting step in glycerol production is the transition from DHAP to glycerol-3-phosphate (Blomberg and Adler 1989; Remize, Barnavon et al. 2001; Cronwright, Rohwer et al. 2002). The conversion of DHAP to glycerol-3-phosphate is catalysed by the NAD^+ dependent glycerol-3-phosphate dehydrogenase, which also exists in two isoforms: Gpd1 and Gpd2, encoded by two isogenes, *GPD1* and *GPD2*. Gpd1 is located in the cytoplasm (Larsson, Ansell et al. 1993; Albertyn, Hohmann et al. 1994; Valadi, Granath et al. 2004) but can also be found in the peroxysomes (Valadi, Granath et al. 2004), whereas Gpd2 localises in the cytoplasm as well as in the mitochondria (Albertyn, Hohmann et al. 1994; Valadi, Granath et al. 2004). Gpd1 acts mainly together with Gpp2 (alias Hor2); their main function is osmo-protective, whereas Gpd2 in combination with Gpp1 (alias Rhr2) are active mostly under anaerobic conditions (Albertyn, Hohmann et al. 1994; Ansell, Granath et al. 1997; Påhlman, Granath et al. 2001; Remize, Barnavon et al. 2001). The double deletion mutant *gpd1Δgpd2Δ* does not produce glycerol and is highly sensitive to osmotic stress, anaerobic conditions and

also to oxidative stress (Påhlman, Granath et al. 2001; Remize, Cambon et al. 2003). Intracellular levels of the NAD^+ dependent glycerol-3-phosphate dehydrogenase are regulated by the amount of glucose (Sprague and Cronan 1977), redox state and osmolarity (Blomberg and Adler 1989) of the external environment; these three types of regulation occur independently from each other (Albertyn, Hohmann et al. 1994).

The Gpd1 protein has a molecular weight of 42 kDa (Albertyn, van Tonder et al. 1992) and a length of 391 amino acids. It employs NADH as a cofactor, which cannot be replaced by NADPH (Merkel, Straume et al. 1982; Albertyn, van Tonder et al. 1992). *In vitro* Gpd1 catalyses the oxidation of glycerol-3-phophate to dihydroxyacetone phosphate, but *in vivo* the equilibrium is strongly shifted towards glycerol-3-phosphate production (Merkel, Straume et al. 1982). The enzyme activity can raise 10 fold within 2 hours after the osmotic shock but the exact value depends on the particular yeast strain and on the type of osmoticum used (Albertyn, Hohmann et al. 1994). The activity increase seems to be due to the protein amount rather than its activation (Andre, Hemming et al. 1991).

The expression of *GPD1* is controlled by a HOG-dependent response mechanism, although it does not seem to be the only mechanism controlling *GPD1* expression under stress (Albertyn, Hohmann et al. 1994b; Remize, Cambon et al. 2003; Dihazi, Kessler et al. 2004). The basis for Hog1-independent stimulation of *GPD1* expression after osmotic shock, which is about 20% of the full induction, remains unknown. The expression of the *GPD1* gene also appears to be controlled by glucose repression (Andre, Hemming et al. 1991; Albertyn, Hohmann et al. 1994). Over-expression of *GPD1* increases glycerol production (Remize, Barnavon et al. 2001). The *gpd1Δ* mutant produces reduced amounts of glycerol and shows high sensitivity to osmotic stress however the glycerol synthesis is not completely abolished (Albertyn, Hohmann et al. 1994b), due to the presence of *GPD2*.

The Gpd2 protein has molecular weight of 49 kDa. Its polypeptide chain has 384 amino acids in length and shares 69% identity with Gpd1. Especially the central parts of the two proteins show a high degree of similarity (Eriksson, Andre et al. 1995). The activity of Gpd2 is not affected by osmotic stress (Albertyn, Hohmann et al. 1994) but is strongly increased under anaerobic conditions (Valadi, Granath et al. 2004). The molecular mechanisms for this effect are unknown.

Over-expressing the *GPD2* gene enhances glycerol production with equal effectiveness as increased expression of *GPD1* (Remize, Barnavon et al. 2001). It was postulated by Ansell that *GPD1* and *GPD2* can replace each other, both under anaerobic conditions and under hyperosmotic stress (Ansell, Granath et al. 1997), if ectopically expressed. It seems that only strong over-expression of *GPD1* can substitute for the lack of *GPD2* in mutants with respiratory defects (Valadi, Granath et al. 2004). Moreover, the increase in glycerol production caused by stimulation of *GPD2* expression in anaerobic conditions prepares the cells for hyper-osmotic stress (Krantz, Nordlander et al. 2004). Summarising, the main function of *GPD2* seems to be associated with maintaining redox balance under anaerobic conditions, which is achieved by re-oxidizing NADH (Ansell, Granath et al. 1997; Costenoble, Valadi et al. 2000).

The rate of glycerol production increases significantly upon hyper osmotic stress. This effect is especially strong for cells pre-adapted to the actual osmotic shock by a milder stress treatment. Pre-conditioning with low concentrations of osmoticum also improves the viability of the osmotically stressed cells (Blomberg and Adler 1989). The amount of glycerol

accumulated inside the cells depends on the concentration of the stress agent and on the particular yeast strain (Albertyn, Hohmann et al. 1994).

4.3.2. Stimulation of glycolysis

Another mechanism apparently contributing to glycerol accumulation is the activity of the 6-phopshofructo-2-kinase. This enzyme, whose activity was reported to be stimulated by the HOG pathway (Dihazi, Kessler et al. 2004) (and is also affected by PKA and Slt2), catalyses the production of fructose-2,6-diphosphate from fructose-6-monophosphate and ATP (Hers and Van Schaftingen 1982). Fructose-2,6-diphosphate in turn stimulates the activity of 6-phosphofructo-1-kinase, Pfk1 (Hers and Van Schaftingen 1982), which is a key glycolytic enzyme acting upstream of the branching point to glycerol.

In baker's yeast there are two isoforms of the 6-phosphofructo-2-kinase: Pfk26 and Pfk27. Nomenclature is somewhat confusing as 6-phosphofructo-2-kinase is sometimes referred to as Pfk2 (Hers and Van Schaftingen 1982; Francois, Van Schaftingen et al. 1984; Dihazi, Kessler et al. 2004). In yeast, however, the 6-phosphofructo-1-kinase, (referred to as Pfk1) and consisting of alpha and beta subunits, is encoded by the genes *PFK1* and *PFK2* (SGD ; Clifton and Fraenkel 1982; Kretschmer and Fraenkel 1991). In this work I used the genetic nomenclature, i.e. Pfk1 and Pfk2 for 6-phosphofructo-1-kinase and Pfk26 and Pfk27 for 6-phosphofructo-2-kinase (Rider, Bertrand et al. 2004).

Pfk26, encoded by the *PFK26* gene, is 827 amino acids long and has a molecular weight of 93.5 kDa. This is almost twice the size of its 55 kDa mammalian orthologue (Kretschmer and Fraenkel 1991). The amino acid sequence of Pfk26 shows 42% identity with the rat liver bi-functional 6-phosphofructo-2-kinase/fructose-2,6-diphosphatase. The yeast Pfk26 lacks phosphatase activity, in spite of high similarity to the mammalian homologue in the phosphatase domain. The reason for this is the substitution of a histidine residue (His258 in rat liver), which acts as a phosphate acceptor in the diphosphatase reaction, to a serine (Ser404 in yeast). However, the serine residue, which acts as a proton donor in the diphosphatase reaction, is present (Kretschmer and Fraenkel 1991). The exchange of Ser-404 to His-404 turns Pfk26 into a bi-functional 6-phosphofructo-2-kinase/fructose-2,6-diphosphatase (Kretschmer, Langer et al. 1993), resembling the bi-functional mammalian enzyme.

The activity of Pfk26 increases in the presence of glucose (Clifton and Fraenkel 1982; Francois, Van Schaftingen et al. 1984). Glucose in the medium activates the Ras-adenylate cyclise pathway leading to activation of the cAMP-dependent protein kinase (PKA), which phosphorylates Pfk26. Yeast 6-phosphofructo-2-kinase is activated by phosphorylation, in contrary to the liver enzyme, which is inactivated in this way (Francois, Van Schaftingen et al. 1984). Pfk26 occurs in an inactive form when cells are grown on ethanol (Goncalves, Griffioen et al. 1997); and it is activated by the PKA pathway after switching to fermentative conditions (Francois, Van Schaftingen et al. 1984). Thus, Pfk26 seems crucial for the rapid increase in fructose-2,6-diphosphate levels directly after the carbon source change (Goncalves, Griffioen et al. 1997).

PFK26 mRNA occurs in large amounts in respiring cells. The transcriptional regulation of both the *PFK26* and *PFK27* genes appears to depend not only on changes in PKA activity, but

also on an alternative pathway (perhaps the TOR pathway), which would regulate the catalytic subunits of PKA (Goncalves, Griffioen et al. 1997). Results from Pfk26 phosphorylation studies also suggest the existence of at least another kinase, apart from PKA (Dihazi, Kessler et al. 2003). This may be Hog1: the 6-phosphofructo-2-kinase is activated upon increased external osmolarity, due to its phosphorylation via the HOG pathway (Dihazi, Kessler et al. 2004).

Pfk27, encoded by the *PKF27* gene, is 397 amino acids long and has a molecular weight of 45.3 kDa. The amino acid sequence resembles only the kinase domain of bi-functional 6-phosphofructo-2-kinase/fructose-2,6-diphosphatases (Boles, Gohlmann et al. 1996), while the phosphatase domain is not present in this protein (Rider, Bertrand et al. 2004). The deletion mutant *pfk27Δ* has a similar level of fructose-2,6-diphosphate as in the wild type, due to the presence of the other 6-phosphofructo-2-kinase isoenzyme, Pfk26 (Boles, Gohlmann et al. 1996).

Fructose-2,6-diphosphate is not a glycolytic intermediate but controls glycolysis. It stimulates the activity of 6-phospho-fructo-1-kinase, the enzyme that catalyses the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate (Hers and Van Schaftingen 1982; Nissler, Otto et al. 1983), and it inhibits the corresponding phosphatase, fructose-1,6-bisphosphatase (Hers and Van Schaftingen 1982; Boles, Gohlmann et al. 1996). Interestingly, increasing the concentration of fructose-2,6-diphosphate along with 6-phosphofructo-1-kinase activity does not seem to result in an increase of the glycolytic flux (Muller, Zimmermann et al. 1997). When grown on glucose, yeast cells contain micro molar concentrations of fructose-2,6-diphosphate, while the compound is undetectable during growth on non-glucose carbon sources (Lederer, Vissers et al. 1981; Hers and Van Schaftingen 1982). Double deletion mutants (*pfk26Δpfk27Δ*) are unable to synthesise fructose-2,6-diphosphate. The consequence is slight delay in the initial phase of growth after glucose addition, even though fructose-2,6-diphosphate was proposed to be the predominant effector on glycolysis in *S. cerevisiae* (Boles, Gohlmann et al. 1996).

The hydrolysis of Fru-2,6-DP to fructose-6-phosphate and Pi, is catalysed by the fructose-2,6-bisphosphatase (Fbp26). This reaction involves a histidine residue as an acceptor for the phosphate group (Paravicini and Kretschmer 1992). Fructose-2,6-diphosphate also seems to be dephosphorylated by an unspecific phosphatases. Fbp26 is a mono-functional enzyme, derived from the same bi-functional 6-phosphofructo-2-kinase/fructose-2,6-diphosphatase as Pfk26 and Pfk27 (Rider et al. 2004). Fructose-2,6-bisphosphatase has 452 amino acids and a molecular weight of 52.7 kDa (Paravicini and Kretschmer 1992).

4.3.3. Control of glycerol export

Even under normal osmolarity *S. cerevisiae* cells growing with a sugar as carbon source constantly produce glycerol in order to maintain a correct redox balance. In the absence of hyperosmotic stress, the excess glycerol leaks out freely through a glycerol facilitator, Fps1, present in the plasma membrane. Cells lacking Fps1 adapt to increased external osmolarity normally. However, upon hypo-osmotic stress most of *fps1Δ* cells die (Tamas, Luyten et al. 1999). Such mutants also are impaired when glycerol is overproduced due to over-expression of *GPD1* (Luyten, Albertyn et al. 1995; Remize, Barnavon et al. 2001). Moreover, *fps1Δ*

mutants have a cell fusion defect during mating probably because turgor needs to be temporarily reduced in this situation (Philips and Herskowitz 1997).

Glycerol accumulation after hyperosmotic shock seems to be ensured by rapid closure of Fps1. Mutants exist in which closure of Fps1 seems to be defective resulting in leakage of the glycerol to the medium, delayed and incomplete glycerol accumulation, glycerol overproduction and sensitivity to hyperosmotic stress (Tamas, Luyten et al. 1999). The detailed mechanisms that control Fps1 and the exact involvement of Hog1 remain to be elucidated. Recently, several proteins interacting with Fps1 have been reported but also their role is not clear at the moment ((Beese, Negishi et al. 2009), Hohmann lab, unpublished data).

Fps1 denotes Fdp1 supressor. The protein is 669 amino acids long and has a predicted molecular weight of 74 kDa. Fps1 is a member of the aquaporin/aquaglyceroporin family (Luyten, Albertyn et al. 1995). Consequently, the structure of Fps1 consists of 6 trans-membrane domains linked with loops, which are denoted from A to E. Loop B and E are directly involved in the glycerol channel formation (Bill, Hedfalk et al. 2001). The amino acid chain has long hydrophilic extensions, located at the inner side of the plasma membrane, which seem to have regulatory role: truncation results in high unregulated glycerol transport.

As a glycerol facilitator, Fps1 allows glycerol diffusion in both directions: into the cell and outwards, depending on the conditions. Fps1 does not seem to be required for glycerol uptake when this polyol is used as a source for carbon and energy (this role seems to be taken by Stl1) but instead the role of Fps1 seems to be focused on osmoregulation (Tamas, Luyten et al. 1999).

4.3.4. Glycerol uptake from the exterior

Increasing the amount of glycerol inside the cell can also be achieved by importing it from outside via Stl1 (Ferreira, van Voorst et al. 2005). Stl1 is a protein located in the plasma membrane. It belongs to the sugar transporter family (STL1 – Sugar Transporter Like) and acts as a glycerol/proton symporter. Glycerol/proton symport from the external environment is subject to glucose repression (Lages and Lucas 1997).

The *STL1* gene is strongly up-regulated under hyper-osmotic conditions and this up-regulation depends completely on the HOG pathway activity. Consequently, the level of Stl1 in the cell increases strongly under hyperosmotic conditions (Ferreira, van Voorst et al. 2005). Upon deletion of *HOT1*, a gene encoding one of the HOG-dependent transcription factors, the induction of *STL1* expression is completely abolished (Rep, Krantz et al. 2000). Mutants lacking *STL1* are unable to utilise glycerol as a source of carbon or energy (Ferreira, van Voorst et al. 2005).

4.4. Other osmotic protectors

Polyols are prominent among compounds accumulated by fungi in response to hyper-osmotic stress to restore water influx. From the chemical point of view, polyols, or sugar alcohols, polyhydric alcohols or polyalcohols, are neither alcohols nor sugars. They are organic compounds, containing a hydroxyl group on each carbon atom, obtained by reducing the carbonyl group of a carbohydrate to an -OH group. Polyhydric alcohols are resistant to enzymatic action, microbial attack, or yeast fermentation; they are chemically stable under various conditions, including heat and wide ranges of pH. Used in food industry as sweeteners, most importantly polyols are osmotically active; therefore many organisms employ them as compatible solutes (Yancey, Clark et al. 1982; Yancey 2005). Glycerol is accumulated by *Saccharomyces cerevisiae* and other yeasts.

However, *Saccharomyces cerevisiae* also accumulates trehalose. Trehalose is a twelve-carbon disaccharide, constituting of two glucose molecules bound with an α,α-1,1-glucoside bond. Although the main function of trehalose lies in protecting enzymes from heat inactivation and preventing the desiccation of membranes, it contributes also to the intracellular osmotic pressure. It was observed that at high concentrations (above 1 M) trehalose and glycerol have a synergistic osmotic effect instead of a purely additive one: the resulting osmolarity is higher than that predicted from the sum of their concentrations (Davis, Burlak et al. 2000). Even though at physiological concentrations this effect does not play a role, the combination of glycerol and trehalose is still beneficial under hyper-osmotic conditions. It has been postulated that trehalose might be important for osmotic regulation in resting cells, which do not seem to accumulate glycerol (Hounsa, Brandt et al. 1998).

4.4.1. Polyols as a tool for studying HOG response characteristics

Polyols can be used as tool to study osmotic stress response characteristics. A semi-artificial system for controlling the HOG pathway activation period was designed by Karlgren and co-workers (Karlgren, Pettersson et al. 2005). The system includes a mutant unable to produce glycerol, an artificial adaptation mechanism and a stress inductor. The mutant lacks both isoforms of the NAD+-dependent glycerol-3-phosphate dehydrogenase, Gpd1 and Gpd2 (*gpd1Δgpd2Δ*), the artificial adaptation is achieved by over-expressing a constitutively open aquaglyceroporin, Fps1-Δ1 or rat AQP9, and stress is induced by treating the cells with polyols of different sizes: glycerol (3C), erythritol (4C), xylitol (5C) and sorbitol (6C). If the size of the polyol is sufficiently small, it can pass through the lumen of the aquaglyceroporin, thereby compensate for the osmotic stress and mimicking natural adaptation. The uptake rate through the glycerol facilitator is reversely proportional to the size of the polyol, which results in a time-varying HOG activation. In this way, the period of the HOG pathway activation can be studied. An extended study using this system, including microarrays and single cell analysis, is presented in Paper V.

5. Conclusions

In Paper I we have presented a mathematical model, which describes the biophysical and mechanical properties of *S. cerevisiae* cells upon hyperosmotic stress. We have measured minimal cell volumes for a wide range of external osmolarities, in order to infer the turgor pressure and volumetric elastic modulus for each given condition. We found that turgor pressure is negligible at a relative cell volume of approximately 93 % and that, for most stress conditions, the cell wall tightly follows the cell membrane on shrinkage, indicating that its elasticity is rather high. We also could not reject the hypothesis of a direct 1:1 relationship between turgor loss and HOG pathway activation. The latter issue was further investigated in Paper III.

In Paper II we continued studying the relation between cell volume and external osmolarity, and extended our investigations also to nuclear volume and cell volume recovery over time. We postulate that the cytoplasm could play a protective role for the nucleus for high stress levels (above 600 mM NaCl). We described the technological advancements made for obtaining our experimental contribution to Paper I, as well as their development, which was necessary for time-resolved measurements of cellular and nuclear volume changes. Data analysis was highly facilitated due to a specially tailored image analysis software (CellStress (Smedh, Beck et al. 2010)). We found that immobilising yeast cells on a pre-treated surface is more suitable for cell volume measurements than keeping them inside an optical trap throughout the experiment.

The work presented in Paper III employed the same setup and image analysis software as Paper II and was dedicated to testing the hypothesis of the direct 1:1 relationship between turgor loss and HOG pathway activation indicated by Paper I. We found that the latter correlation holds only for osmotic stresses up to 600 mM NaCl. A delay in Hog1 nuclear retention was observed in cells treated with 800 mM and 1000 mM NaCl – the same concentrations, for which we concluded that the cytoplasm facilitates nuclear volume recovery in Paper II. Experiments to be performed before submission to a journal will include a comparison of Hog1 nucleo-cyoplasmic shuttling properties with those of another protein which also changes its sub-cellular localisation in response to hyper-osmotic stress, namely Msn2.

In Paper IV we investigated Hog1 nucleo-cyotplasmic shuttling and cell volume recovery, as well as changes in gene expression, for a conditional osmotic stress system, employing polyols of different sizes. From the single cell experiments we found that, at a given stress level, a longer period of adaptation results in a delay in both processes. This time the level of stress depended not on the *osmoticum* concentration (which was constant) but of the size of the polyol used as hyper-osmotic agent. These observations indicate that certain adaptive responses must occur inside the cell to make it signalling competent. The nature of those responses and how they couple to other osmotic recovery processes is presently unclear.

Papers V and VI are dedicated to investigating the consequences of the HOG pathway activity and its link to cellular metabolism. Paper V is a purely theoretical analysis of the response coefficients, based on previous theoretical work and existing experimental data. In this paper we found that, depending on initial relations between glycerol and pyruvate production, the increased glycerol production can have a substantial negative effect on the pyruvate production rate. The time-varying response coefficients reflect sensitivities of species

concentrations to parameter variations. Using this approach we predicted that the activation of Pfk26 by Hog1 leads to a significantly increased glycolytic flux during osmotic adaptation and to a decelerated adaptation in case of Pfk26 knockout. This prediction was further tested in Paper VI.

Paper VI extends the quantitative investigation of the different mechanisms contributing to glycerol accumulation, and their temporal characteristics, also using response coefficients. Based on large set of experimental data, we iteratively combined experimentation and modelling to establish the temporal importance of each glycerol accumulation mechanism. We tested the robustness of the overall glycerol accumulation process to various perturbations, "fail-on" and "fail-off". The results confirmed the influence of Hog1 on the glycolytic flux postulated in Paper V, indicated that deleting each glycerol accumulation mechanism prolongs the period of Hog1 activation and suggested the existence of a direct interaction between Hog1 and Fps1.

In summary, in this thesis I investigated the quantitative aspects of yeast osmotic regulation. The work provides precise, time resolved information about the biophysical characteristics of osmotic regulation, such as cell volume and turgor loss and recovery, which have not been investigated in a systematic and quantitative manner in the past. Our work also provides new insight into the network properties of the HOG pathway, indicating the limitations of the response linearity range. The work which I present here contributed also to the development of the single cell analysis platform, dedicated to analysing sub-cellular protein shuttling, correlated with measurements of cellular and nuclear volume. I have also produced yeast mutants which enable this kind of observations. Another important contribution of this thesis is the quantitative characterisation of the consequences of HOG activity, meaning the interdependence of different mechanisms of glycerol accumulation.

6. Acknowledgements

This thesis would have never been created, if not the help of numerous people. I will surely not manage to mention all the persons, whom I owe my gratitude. However, I will allow myself to state by name at least some of the people, without whom I would surely not manage to finalise this work.

First of all I would like to thank my supervisor, Stefan Hohmann, as well as my co-supervisors, Edda Klipp and Mattias Goksör. I am extremely grateful for your support, patience and valuable input.

To the other professors in the Department, especially Thomas Nyström, Anders Blomberg, Lennart Adler and Per Sunnerhagen – thank you for creating the friendly and intellectually stimulating atmosphere.

To Ellinor Pettersson – simply: thank you! ☺

My closest co-workers, Emma Eriksson, Caroline Beck and Clemens Kühn, thank you for all the long hours spent together in and outside the lab(s).

To my room and table mates, Dagmara Klein, Jimmy Kjellen, Calle Tiger and Nina Pettersson – thank you for the nice company and interesting discussions, on diverse topics. ☺

I would like to thank Markus Tamas – for the scientific advice and, most of all, for the memorable trip to the Yeast Geneteics 2005 conference. The latter concerns also Dagmara and Yujun: Dear Golden Tank Crew – if you need a map reader, count on me! ☺

Thanks also to my elder and younger colleagues, present and past group members: Bodil, Karin L., Marcus K., Karin E., Jenny, Therese, Lasse, Kentaro, Raul, Cecilia, Roja, Soodeh, Sylwia, Madde, Tian, Maria E., Avi, Ivan, Michael T, Martin M, Caroline F, Yulia and Yujun.

Special thanks to the Rescue Team: Nika, Dani, Calle (once again), Dominik and Manely, who helped me with shopping, laundry, garbage and bike rescuing, when I was unable to move.

I owe my great gratitude to Takako and Peter, without whose help lab life would have been very hard. For the same reason, I also thank the support and technical staff in the building, especially Rigmor, Liliana, Zaklina, Bruno, Lars and Leif.

I would like to thank also members of other groups, whom I had the pleasure to meet and work, exchange experiences and/or socialise with: Maria Smedh, Kristin Sott, Charlotte Hamngren, Christian Waltermann, Maria Cvijovic, Janeli Sarv, Jonathan Esguerra, Luciano Fernandez, Annabelle Forsmark, Mats Hulander, Manish Rauthan, Sourav Datta, Karen Brackley, John Patrick Alao and all the other colleagues from Lundberg and outside. In case I forgot someone: thank you as well. ☺

Special thanks to Jonas Carlsson, not only for the computer help!

Last but not least, my dear friend Doryaneh – thank you for being there and being who you are. ☺

I would also like to express my gratitude to all the persons back in Poland, my home country, for their encouragement and support. Here the first and biggest thanks I owe to my Parents, without whose upbringing I possibly wouldn't ever decide to become a scientist.
Kochani, bardzo Wam dziękuję!

A great thanks to Mrs Marzanna Wikiera – my biology teacher, due to whose influence I decided to study biology, and to Dr Przemyslaw Plonka – my first supervisor, thanks to whom I became interested in interdisciplinary research.

To all the other Near and Dear – thank you for your warm thoughts and support

And (again) last but not least: Darek – my beloved husband, for everything.
Dziękuję Ci, Kochanie!

7. References

Abramowitz, M. (2003). Microscope. Basics and beyond, Olympus Amreica Inc.

Ajo-Franklin, C. M., D. A. Drubin, et al. (2007). "Rational design of memory in eukaryotic cells." Genes Dev **21**(18): 2271-2276.

Alberghina, L. and D. Porro (1993). "Quantitative flow cytometry: analysis of protein distributions in budding yeast. A mini-review." Yeast **9**: 815-823.

Albertyn, J., S. Hohmann, et al. (1994). "Characterization of the osmotic-stress response in Saccharomyces cerevisiae: osmotic stress and glucose repression regulate glycerol-3-phosphate dehydrogenase independently." Curr Genet **25**(1): 12-18.

Albertyn, J., S. Hohmann, et al. (1994b). "GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway." Mol Cell Biol **14**(6): 4135-4144.

Albertyn, J., A. van Tonder, et al. (1992). "Purification and characterization of glycerol-3-phosphate dehydrogenase of Saccharomyces cerevisiae." FEBS Lett **308**(2): 130-132.

Alepuz, P. M., A. Jovanovic, et al. (2001). "Stress-induced map kinase Hog1 is part of transcription activation complexes." Mol Cell **7**(4): 767-777.

Alon, U. (2007). "Network motifs: theory and experimental approaches." Nat Rev Genet **8**(6): 450-461.

Alon, U. (2007). "Simplicity in biology." Nature **446**(7135): 497.

Ammerer, G. (1994). "Sex, stress and integrity: the importance of MAP kinases in yeast." Curr Opin Genet Dev **4**(1): 90-95.

Andre, L., A. Hemming, et al. (1991). "Osmoregulation in Saccharomyces cerevisiae. Studies on the osmotic induction of glycerol production and glycerol-3-phosphate dehydrogenase (NAD+)." FEBS Lett **286**(1-2): 13-17.

Ansell, R., K. Granath, et al. (1997). "The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by GPD1 and GPD2 have distinct roles in osmoadaptation and redox regulation." Embo J **16**(9): 2179-2187.

Ashkin, A. (1997). "Optical trapping and manipulation of neutral particles using lasers." Proc Natl Acad Sci U S A **94**(10): 4853-4860.

Ashkin, A. and J. M. Dziedzic (1987). "Optical trapping and manipulation of viruses and bacteria." Science **235**(4795): 1517-1520.

Ashkin, A., J. M. Dziedzic, et al. (1986). "Observation of a single-beam gradient force optical trap for dielectric particles." Opt Lett **11**(5): 288.

Atkins, P. W. (1998). The Second Law: the machinery. Physical Chemistry**:** 132.

Atkins, P. W. (1998). Simple mixtures. Physical Chemistry**:** 180-186.

Becksei, A. and L. Serrano (2000). "Engineering stability in gene networks by autoregualtion." Nature **405**(6786): 590-593.

Beebe, D. J., G. A. Mensing, et al. (2002). "Physics and applications of microfluidics in biology." Annu Rev Biomed Eng **4**: 261-286.

Beese, S. E., T. Negishi, et al. (2009). "Identification of positive regulators of the yeast fps1 glycerol channel." PLoS Genet **5**(11): e1000738.

Bill, R. M., K. Hedfalk, et al. (2001). "Analysis of the pore of the unusual major intrinsic protein channel, yeast Fps1p." J Biol Chem **276**(39): 36543-36549.

Blomberg, A. and L. Adler (1989). "Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD+) in acquired osmotolerance of Saccharomyces cerevisiae." J Bacteriol **171**(2): 1087-1092.

Boles, E., H. W. Gohlmann, et al. (1996). "Cloning of a second gene encoding 5-phosphofructo-2-kinase in yeast, and characterization of mutant strains without fructose-2,6-bisphosphate." Mol Microbiol **20**(1): 65-76.
Brewster, J. L., T. de Valoir, et al. (1993). "An osmosensing signal transduction pathway in yeast." Science **259**(5102): 1760-1763.
Brown, A. D. (1974). "Microbial water relations: features of the intracellular composition of sugar-tolerant yeasts." J Bacteriol **118**(3): 769-777.
Bruggeman, F. J. and H. V. Westerhoff (2007). "The nature of systems biology." Trends in Microbiology **15**(1): 45-50.
Caffrey, D. R., L. A. O'Neill, et al. (1999). "The evolution of the MAP kinase pathways: coduplication of interacting proteins leads to new signaling cascades." J Mol Evol **49**(5): 567-582.
Choi, M. Y., G. Y. Kang, et al. (2008). "Analysis of dual phosphorylation of Hog1 MAP kinase in Saccharomyces cerevisiae using quantitative mass spectrometry." Mol Cells **26**(2): 200-205.
Clifton, D. and D. G. Fraenkel (1982). "Mutant studies of yeast phosphofructokinase." Biochemistry **21**(8): 1935-1942.
Clotet, J., X. Escote, et al. (2006). "Phosphorylation of Hsl1 by Hog1 leads to a G2 arrest essential for cell survival at high osmolarity." Embo J **25**(11): 2338-2346.
Cobb, M. H. and E. J. Goldsmith (1995). "How MAP kinases are regulated." J Biol Chem **270**(25): 14843-14846.
Cooper, J. A. (1994). "MAP kinase pathways. Straight and narrow or tortuous and intersecting?" Curr Biol **4**(12): 1118-1121.
Cosgrove, D. J. (1988). "In defence of the cell volumetric elastic modulus." Plant Cell Environ **11**: 67-69.
Costenoble, R., H. Valadi, et al. (2000). "Microaerobic glycerol formation in Saccharomyces cerevisiae." Yeast **16**(16): 1483-1495.
Creager, A. N. and H. Landecker (2009). "Technical matters: method, knowledge and infrastructure in twentieth-century life science." Nat Methods **6**(10): 701-705.
Cronwright, G. R., J. M. Rohwer, et al. (2002). "Metabolic control analysis of glycerol synthesis in Saccharomyces cerevisiae." Appl Environ Microbiol **68**(9): 4448-4456.
Darzynkiewicz, Z., E. Bedner, et al. (1999). "Laser-scanning cytometry: a new instrumentation with many applications." Experimental Cell Research **249**(1): 1-12.
Davis, D. J., C. Burlak, et al. (2000). "Osmotic pressure of fungal compatible osmolytes." Mycological Research **104**(7): 800-804.
de Nadal, E. and F. Posas (2010). "Multilayered control of gene expression by stress-activated protein kinases." Embo J **29**(1): 4-13.
de Nadal, E., F. X. Real, et al. (2007). "Mucins, osmosensors in eukaryotic cells?" Trends Cell Biol **17**(12): 571-574.
Dean, P. N. and R. A. Hoffman (2007). "Overview of flow cytometry instrumentation." Curr Protoc Cytom **Chapter 1**: Unit1 1.
Deptala, A., E. Bedner, et al. (2001). "Unique analytical capabilities of laser scanning cytometry (LSC) that complement flow cytometry." Folia Histochemica et Cytobiologica **39**(2): 87-89.
Dhillon, A. S., S. Hagan, et al. (2007). "MAP kinase signalling pathways in cancer." Oncogene **26**(22): 3279-3290.
Di Carlo, D. and L. P. Lee (2006). "Dynamic single-cell analysis for quantitative biology." Anal Chem **78**(23): 7918-7925.

Dihazi, H., R. Kessler, et al. (2003). "Glucose-induced stimulation of the Ras-cAMP pathway in yeast leads to multiple phosphorylations and activation of 6-phosphofructo-2-kinase." Biochemistry **42**(20): 6275-6282.

Dihazi, H., R. Kessler, et al. (2004). "High osmolarity glycerol (HOG) pathway-induced phosphorylation and activation of 6-phosphofructo-2-kinase are essential for glycerol accumulation and yeast cell proliferation under hyperosmotic stress." J Biol Chem **279**(23): 23961-23968.

Drogen, F., S. M. O'Rourke, et al. (2000). "Phosphorylation of the MEKK Ste11p by the PAK-like kinase Ste20p is required for MAP kinase signaling in vivo." Curr Biol **10**(11): 630-639.

Ehrenberg, M., J. Elf, et al. (2009). "Systems Biology: Nobel Symposium 146." FEBS Lett **583**(24): 3381.

Elowitz, M. B., A. J. Levine, et al. (2002). "Stochastic gene expression in a single cell." Science **297**(5584): 1183-1186.

Enger, J., M. Goksör, et al. (2004). "Optical tweezers applied to a microfluidic system." Lab Chip **4**: 196-200.

Eriksson , E. (2009). Towards quantitative single cell analysis using optical tweezers and microfluidics. Department of Physics. Göteborg, Sweden, University of Gothenburg. **PhD**.

Eriksson, E., J. Enger, et al. (2007). "A microfluidic system in combination with optical tweezers for analyzing rapid and reversible cytological alterations in single cells upon environmental changes." Lab Chip **7**(1): 71-76.

Eriksson, E., K. Sott, et al. (2010). "A microfluidic device for reversible environmental changes around single cells using optical tweezers for cell selection and positioning." Lab Chip **10**(5): 617-625.

Eriksson, P., L. Andre, et al. (1995). "Cloning and characterization of GPD2, a second gene encoding sn-glycerol 3-phosphate dehydrogenase (NAD+) in Saccharomyces cerevisiae, and its comparison with GPD1." Mol Microbiol **17**(1): 95-107.

Escote, X., M. Zapater, et al. (2004). "Hog1 mediates cell-cycle arrest in G1 phase by the dual targeting of Sic1." Nat Cell Biol **6**(10): 997-1002.

Ferreira, C., F. van Voorst, et al. (2005). "A member of the sugar transporter family, Stl1p is the glycerol/H+ symporter in Saccharomyces cerevisiae." Mol Biol Cell **16**(4): 2068-2076.

Ferrigno, P., F. Posas, et al. (1998). "Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1." Embo J **17**(19): 5606-5614.

Francois, J., E. Van Schaftingen, et al. (1984). "The mechanism by which glucose increases fructose 2,6-bisphosphate concentration in Saccharomyces cerevisiae. A cyclic-AMP-dependent activation of phosphofructokinase 2." Eur J Biochem **145**(1): 187-193.

Freeman, M. (2000). "Feedback control of intracellular signalling in development." Nature **408**(6810): 313-319.

Furukawa, K., Y. Hoshi, et al. (2005). "Aspergillus nidulans HOG pathway is activated only by two-component signalling pathway in response to osmotic stress." Mol Microbiol **56**(5): 1246-1261.

Gancedo, C., J. M. Gancedo, et al. (1968). "Glycerol metabolism in yeasts. Pathways of utilization and production." Eur J Biochem **5**(2): 165-172.

Gennemark, P., B. Nordlander, et al. (2006). "A simple mathematical model of adaptation to high osmolarity in yeast." In Silico Biol **6**(3): 193-214.

Giepmans, B. N. G., S. R. Adams, et al. (2006). "A fluorescent toolbox for assessing protein location and function." Science **312**(217): 217-224.

Giuliano, K. A., R. L. DeBiasio, et al. (1997). "High-content screening: A new approach to easing key bottlenecks in the drug discovery process." Journal of Biomolecular Screening **2**(4): 249 -259.

Goncalves, P. M., G. Griffioen, et al. (1997). "Signalling pathways leading to transcriptional regulation of genes involved in the activation of glycolysis in yeast." Mol Microbiol **25**(3): 483-493.

Gustin, M. C., J. Albertyn, et al. (1998). "MAP kinase pathways in the yeast Saccharomyces cerevisiae." Microbiol Mol Biol Rev **62**(4): 1264-1300.

Harold, F. M. (2002). "Force and compliance: rethinking morphogenesis in walled cells." Fungal Genetics and Biology **37**(3): 271–282.

Hartwell, L. H., J. J. Hopfield, et al. (1999). "From molecular to modular biology." Nature **402**(6761 suppl): C47-C52.

Hers, H. G. and E. Van Schaftingen (1982). "Fructose 2,6-bisphosphate 2 years after its discovery." Biochem J **206**(1): 1-12.

Hersen, P., M. N. McClean, et al. (2008). "Signal processing by the HOG MAP kinase pathway." PNAS **105**(20): 7165-7170.

Hirayama, T., T. Maeda, et al. (1995). "Cloning and characterization of seven cDNAs for hyperosmolarity-responsive (HOR) genes of Saccharomyces cerevisiae." Mol Gen Genet **249**(2): 127-138.

Hohmann, S. (2002). "Osmotic stress signaling and osmoadaptation in yeasts." Microbiol Mol Biol Rev **66**(2): 300-372.

Hohmann, S. (2009). "Control of high osmolarity signalling in the yeast Saccharomyces cerevisiae." FEBS Lett **583**(24): 4025-4029.

Hounsa, C. G., E. V. Brandt, et al. (1998). "Role of trehalose in survival of Saccharomyces cerevisiae under osmotic stress." Microbiology **144**(3): 671-680.

Jass, J., S. Schedin, et al. (2004). "Physical properties of Escherichia coli P pili measured by optical tweezers." Biophys J **87**(6): 4271-4283.

Karlgren, S., N. Pettersson, et al. (2005). "Conditional osmotic stress in yeast: a system to study transport through aquaglyceroporins and osmostress signaling." J Biol Chem **280**(8): 7186-7193.

Kitano, H. (2002). "Computational systems biology." Nature **420**(6912): 206-210.

Kitano, H. (2002b). "Systems biology: a brief overview." Science **295**(5560): 1662-1664.

Klipp, E., W. Liebermeister, et al. (2009). Systems biology: a textbook. Weinheim, Wiley.

Klipp, E., B. Nordlander, et al. (2005). "Integrative model of the response of yeast to osmotic shock." Nat Biotechnol **23**(8): 975-982.

Krantz, M., D. Ahmadpour, et al. (2009). "Robustness and fragility in the yeast high osmolarity glycerol (HOG) signal-transduction pathway." Molecular Systems Biology **5**(281): 1-7.

Krantz, M., E. Becit, et al. (2006). "Comparative genomics of the HOG-signalling system in fungi." Curr Genet **49**(3): 137-151.

Krantz, M., B. Nordlander, et al. (2004). "Anaerobicity prepares Saccharomyces cerevisiae cells for faster adaptation to osmotic shock." Eukaryot Cell **3**(6): 1381-1390.

Kretschmer, M. and D. G. Fraenkel (1991). "Yeast 6-phosphofructo-2-kinase: sequence and mutant." Biochemistry **30**(44): 10663-10672.

Kretschmer, M., C. Langer, et al. (1993). "Mutation of monofunctional 6-phosphofructo-2-kinase in yeast to bifunctional 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase." Biochemistry **32**(41): 11143-11148.

Kuhn, C., E. Petelenz, et al. (2008). "Exploring the impact of osmoadaptation on glycolysis using time-varying response-coefficients." Genome Inform **20**: 77-90.

Lages, F. and C. Lucas (1997). "Contribution to the physiological characterization of glycerol active uptake in Saccharomyces cerevisiae." Biochim Biophys Acta **1322**(1): 8-18.
Larsson, K., R. Ansell, et al. (1993). "A gene encoding sn-glycerol 3-phosphate dehydrogenase (NAD+) complements an osmosensitive mutant of Saccharomyces cerevisiae." Mol Microbiol **10**(5): 1101-1111.
Lavis, L. D. and R. T. Raines (2008). "Bright ideas for chemical biology." ACS Chemical Biology **3**(3): 142-155.
Lederer, B., S. Vissers, et al. (1981). "Fructose 2,6-bisphosphate in yeast." Biochem Biophys Res Commun **103**(4): 1281-1287.
Lichtman, J. W. and J. A. Conchello (2005). "Fluorescence microscopy." Nat Methods **2**(12): 910-919.
Luyten, K., J. Albertyn, et al. (1995). "Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress." Embo J **14**(7): 1360-1371.
Macia, J., S. Regot, et al. (2009). "Dynamic signaling in the Hog1 MAPK pathway relies on high basal signal transduction." Sci Signal **2**(63): ra13.
Maeda, T., M. Takekawa, et al. (1995). "Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor." Science **269**(5223): 554-558.
Maeda, T., S. M. Wurgler-Murphy, et al. (1994). "A two-component system that regulates an osmosensing MAP kinase cascade in yeast." Nature **369**(6477): 242-245.
Maheshri, N. and E. K. O'Shea (2007). "Living with noisy genes: how cells function reliably with inherent variability in gene expression." Annu Rev Biophys Biomol Struct **36**: 413-434.
Marshall, C. J. (1994). "MAP kinase kinase kinase, MAP kinase kinase and MAP kinase." Curr Opin Genet Dev **4**(1): 82-89.
Martin, H., M. Flandez, et al. (2005). "Protein phosphatases in MAPK signalling: we keep learning from yeast." Mol Microbiol **58**(1): 6-16.
Martinez-Pastor, M. T., G. Marchler, et al. (1996). "The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE)." Embo J **15**(9): 2227-2235.
Merkel, J. R., M. Straume, et al. (1982). "Purification and some properties of sn-glycerol-3-phosphate dehydrogenase from Saccharomyces cerevisiae." Anal Biochem **122**(1): 180-185.
Mettetal, J. T., D. Muzzey, et al. (2008). "The frequency dependence of osmo-adaptation in Saccharomyces cerevisiae." Science **319**(5862): 482-484.
Milo, R., S. Shen-Orr, et al. (2002). "Network motifs: simple building blocks of complex networks." Science **298**(5594): 824-827.
Miyawaki, A., A. Sawano, et al. (2003). "Lighting up cells: labelling proteins with fluorophores." Nat Cell Biol **5**(S1): S1-S7.
Muller, S., F. K. Zimmermann, et al. (1997). "Mutant studies of phosphofructo-2-kinases do not reveal an essential role of fructose-2,6-bisphosphate in the regulation of carbon fluxes in yeast cells." Microbiology **143 (Pt 9)**: 3055-3061.
Murphy, D. B., D. W. Piston, et al. (2004-2009). "Spectral bleed-through artefacts in confocal microscopy." Retrieved Aug 2, 2010, from www.olympusfluoview.com/theory/bleedthrough.html.
Nissler, K., A. Otto, et al. (1983). "Similarity of activation of yeast phosphofructokinase by AMP and fructose-2,6-bisphosphate." Biochem Biophys Res Commun **111**(1): 294-300.
Norbeck, J., A. K. Påhlman, et al. (1996). "Purification and characterization of two isoenzymes of DL-glycerol-3-phosphatase from Saccharomyces cerevisiae.

Identification of the corresponding GPP1 and GPP2 genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway." J Biol Chem **271**(23): 13875-13881.

O'Rourke, S. M. and I. Herskowitz (1998). "The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in Saccharomyces cerevisiae." Genes Dev **12**(18): 2874-2886.

O'Rourke, S. M. and I. Herskowitz (2002). "A third osmosensing branch in Saccharomyces cerevisiae requires the Msb2 protein and functions in parallel with the Sho1 branch." Mol Cell Biol **22**(13): 4739-4749.

O'Rourke, S. M. and I. Herskowitz (2004). "Unique and redundant roles for HOG MAPK pathway components as revealed by whole-genome expression analysis." Mol Biol Cell **15**(2): 532-542.

O'Rourke, S. M., I. Herskowitz, et al. (2002). "Yeast go the whole HOG for the hyperosmotic response." Trends Genet **18**(8): 405-412.

Paliwal, S., C. J. Wang, et al. (2008). "Pulsing cells: how fast is too fast?" HFSP Journal **2**(5): 251-256.

Paravicini, G. and M. Kretschmer (1992). "The yeast FBP26 gene codes for a fructose-2,6-bisphosphatase." Biochemistry **31**(31): 7126-7133.

Parkinson, J. S. and E. C. Kofoid (1992). "Communication modules in bacterial signaling proteins." Annu Rev Genet **26**: 71-112.

Pepperkok, R. and J. Ellenberg (2006). "High-throughput fluorescence microscopy for systems biology." Nature Reviews Molecular Cell Biology **7**(690-696).

Philips, J. and I. Herskowitz (1997). "Osmotic balance regulates cell fusion during mating in Saccharomyces cerevisiae." J Cell Biol **138**(5): 961-974.

Posas, F. and H. Saito (1997). "Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK." Science **276**(5319): 1702-1705.

Posas, F. and H. Saito (1998). "Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator." Embo J **17**(5): 1385-1394.

Posas, F., S. M. Wurgler-Murphy, et al. (1996). "Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor." Cell **86**(6): 865-875.

Proft, M., G. Mas, et al. (2006). "The stress-activated Hog1 kinase is a selective transcriptional elongation factor for genes responding to osmotic stress." Mol Cell **23**(2): 241-250.

Proft, M. and K. Struhl (2002). "Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress." Mol Cell **9**(6): 1307-1317.

Påhlman, A. K., K. Granath, et al. (2001). "The yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress." J Biol Chem **276**(5): 3555-3563.

Raser, J. M. and E. K. O'Shea (2004). "Control of stochasticity in eukrayotic gene expression." Science **304**(5678): 1811-1814.

Reiser, V., G. Ammerer, et al. (1999). "Nucleocytoplasmic traffic of MAP kinases." Gene Expr **7**(4-6): 247-254.

Reiser, V., D. C. Raitt, et al. (2003). "Yeast osmosensor Sln1 and plant cytokinin receptor Cre1 respond to changes in turgor pressure." J Cell Biol **161**(6): 1035-1040.

Reiser, V., S. M. Salah, et al. (2000). "Polarized localization of yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42." Nat Cell Biol **2**(9): 620-627.

Remize, F., L. Barnavon, et al. (2001). "Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate limiting for glycerol production in Saccharomyces cerevisiae." Metab Eng **3**(4): 301-312.

Remize, F., B. Cambon, et al. (2003). "Glycerol formation during wine fermentation is mainly linked to Gpd1p and is only partially controlled by the HOG pathway." Yeast **20**(15): 1243-1253.

Rep, M., M. Krantz, et al. (2000). "The transcriptional response of Saccharomyces cerevisiae to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes." J Biol Chem **275**(12): 8290-8300.

Rep, M., M. Proft, et al. (2001). "The Saccharomyces cerevisiae Sko1p transcription factor mediates HOG pathway-dependent osmotic regulation of a set of genes encoding enzymes implicated in protection from oxidative damage." Mol Microbiol **40**(5): 1067-1083.

Rep, M., V. Reiser, et al. (1999). "Osmotic stress-induced gene expression in Saccharomyces cerevisiae requires Msn1p and the novel nuclear factor Hot1p." Mol Cell Biol **19**(8): 5474-5485.

Rider, M. H., L. Bertrand, et al. (2004). "6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis." Biochem J **381**(Pt 3): 561-579.

Saito, H. and K. Tatebayashi (2004). "Regulation of the osmoregulatory HOG MAPK cascade in yeast." J Biochem **136**(3): 267-272.

Schaber, J., M. A. Adrover, et al. (2010). "Biophysical properties of Saccharomyces cerevisiae and their relation to HOG pathway activation." European Biophysics Journal

Schaber, J. and E. Klipp (2008). "Short-term volume and turgor regulation in yeast." Essays Biochem **45**: 147-159.

Schwartz, M. A. and H. D. Madhani (2004). "Principles of MAP kinase signaling specificity in Saccharomyces cerevisiae." Annu Rev Genet **38**: 725-748.

Seydel, C. (2003). "Quantum dots get wet." Science **300**(5616): 80-81.

SGD. "Saccharomyces Genome Database." Retrieved 2010-05-21, from www.yeastgenome.org.

Shaevitz, J. W. (2006, August 22). "A Practical Guide to Optical Trapping." Retrieved Jul 30, 2010, from http://genomics.princeton.edu/shaevitzlab/OT_Practicle_Guide.pdf.

Shaner, N. C., P. A. Steinbach, et al. (2005). "A guide to choosing fluorescent proteins." Nat Methods **2**(12): 905-909.

Shapiro, H. M. (2003). Practical flow cytometry, Wiley-Liss

Smedh, M., C. Beck, et al. (2010). CellStress – open source image analysis program for single-cell analysis. Proceedings SPIE 7762.

Smith, A. E., Z. Zhang, et al. (2000). "The mechanical properties of Saccharomyces cerevisiae." PNAS **97**(18): 9871–9874.

Sott , K., E. Eriksson , et al. (2008). "Optical systems for single cell analyses." Expert Opin Drug Discov **3**(11): 1323-1344.

Sprague, G. F. and J. E. Cronan (1977). "Isolation and characterization of Saccharomyces cerevisiae mutants defective in glycerol catabolism." J Bacteriol **129**(3): 1335-1342.

Stelling, J. (2004). "Mathematical models in microbial systems biology." Curr Opin Microbiol **7**(5): 513-518.

Stock, A. M., V. L. Robinson, et al. (2000). "Two-component signal transduction." Annu Rev Biochem **69**: 183-215.

Svahn, H. A. and A. van den Berg (2007). "Single cells or large populations?" Lab Chip **7**(5): 544 -546.

Tamas, M. J. and S. Hohmann (2003). The osmotic stress response of Saccharomyces cerevisiae. Yeast stress response. Berlin / Heidelberg, Springer. **1:** 121-200

Tamas, M. J., K. Luyten, et al. (1999). "Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation." Mol Microbiol **31**(4): 1087-1104.

Tatebayashi, K., M. Takekawa, et al. (2003). "A docking site determining specificity of Pbs2 MAPKK for Ssk2/Ssk22 MAPKKKs in the yeast HOG pathway." Embo J **22**(14): 3624-3634.

Tatebayashi, K., K. Tanaka, et al. (2007). "Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway." Embo J **26**(15): 3521-3533.

Tatebayashi, K., K. Yamamoto, et al. (2006). "Adaptor functions of Cdc42, Ste50, and Sho1 in the yeast osmoregulatory HOG MAPK pathway." Embo J **25**(13): 3033-3044.

Uesono, Y. and E. A. Toh (2002). "Transient inhibition of translation initiation by osmotic stress." J Biol Chem **277**(16): 13848-13855.

Valadi, A., K. Granath, et al. (2004). "Distinct intracellular localization of Gpd1p and Gpd2p, the two yeast isoforms of NAD+-dependent glycerol-3-phosphate dehydrogenase, explains their different contributions to redox-driven glycerol production." J Biol Chem **279**(38): 39677-39685.

Walker, D. C. and J. Southgate (2009). "The virtual cell - a candidate co-ordinator for 'middle-out' modelling of biological systems." Brief Bioinform **10**(4): 450-461.

Van Wuytswinkel, O., V. Reiser, et al. (2000). "Response of Saccharomyces cerevisiae to severe osmotic stress: evidence for a novel activation mechanism of the HOG MAP kinase pathway." Mol Microbiol **37**(2): 382-397.

Warmka, J., J. Hanneman, et al. (2001). "Ptc1, a Type 2C Ser/Thr Phosphatase, Inactivates the HOG Pathway by Dephosphorylating the Mitogen-Activated Protein Kinase Hog1." Mol Biol Cell **21**(1): 51-60.

West, A. H. and A. M. Stock (2001). "Histidine kinases and response regulator proteins in two-component signaling systems." Trends Biochem Sci **26**(6): 369-376.

Westfall, P. J., J. C. Patterson, et al. (2008). "Stress resistance and signal fidelity independent of nuclear MAPK function." Proc Natl Acad Sci U S A **105**(34): 12212-12217.

Widmann, C., S. Gibson, et al. (1999). "Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human." Physiol Rev **79**(1): 143-180.

Wu, C., G. Jansen, et al. (2006). "Adaptor protein Ste50p links the Ste11p MEKK to the HOG pathway through plasma membrane association." Genes Dev **20**(6): 734-746.

Wurgler-Murphy, S. M., T. Maeda, et al. (1997). "Regulation of the Saccharomyces cerevisiae HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases." Mol Cell Biol **17**(3): 1289-1297.

Yancey, P. H. (2005). "Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses." J Exp Biol **208**(Pt 15): 2819-2830.

Yancey, P. H., M. E. Clark, et al. (1982). "Living with water stress: evolution of osmolyte systems." Science **217**(4566): 1214-1222.

Ye, N., J. Qin, et al. (2007). "Cell-based high content screening using an integrated microfluidic device." Lab Chip **7**(12): 1696 -1704.

Young, C., J. Mapes, et al. (2002). "Role of Ptc2 type 2C Ser/Thr phosphatase in yeast high-osmolarity glycerol pathway inactivation." Eukaryot Cell **1**(6): 1032-1040.

Yuzyuk, T. and D. C. Amberg (2003). "Actin recovery and bud emergence in osmotically stressed cells requires the conserved actin interacting mitogen-activated protein kinase

kinase kinase Ssk2p/MTK1 and the scaffold protein Spa2p." Mol Biol Cell **14**(7): 3013-3026.
Zarrinpar, A., R. P. Bhattacharyya, et al. (2004). "Sho1 and Pbs2 act as coscaffolds linking components in the yeast high osmolarity MAP kinase pathway." Mol Cell **14**(6): 825-832.

Abbreviations

DHAP – Di-hydroxyl-acetone phosphate
Fru-2,6-DP – fructose-2,6-diphosphate
FMN – flavin mononucleotide
G3P – Glycerol-3-phosphate
GFP – Green Fluorescent Protein
HOG – High Osmolarity Glycerol
MAPK – Mitogen Activated Protein Kinase
MAPKK – MAPK Kinase
MAPKKK – MAPKK Kinase
MEK – mitogen extracellular signal-regulated kinase, other term for MAPKK
MEKK – mitogen extracellular signal-regulated kinase kinase, other term for MAPKKK
NAD^+ – Nicotinamide adenine dinucleotide
PKC – Protein Kinase C
SAPK – Stress-Activated Protein Kinase

Gpd1 – NAD^+ dependent glycerol-3-phosphate dehydrogenase
Gpd2 – NAD^+ dependent glycerol-3-phosphate dehydrogenase

Gpp1 – (alias Rhr2) glycerol-3-phosphatase
Gpp2 – (alias Hor2) glycerol-3-phosphatase

Hog1 – High Osmolarity Glycerol response MAPK

Pfk26 – 6-phosphofructo-2-kinase
Pfk27 – 6-phosphofructo-2-kinase