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DISSERTATION

Signalling in space and time:
Catching dynamic interactions using a novel enzymatic tagging approach
in the *S. cerevisiae* HOG pathway

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1 Abstract

Signalling is an important cellular process that requires fast and accurate protein interactions to efficiently transmit signals that ultimately lead to a cellular response, ensuring cell survival. Furthermore, the constant changes of environmental conditions raise the need for a cell to be able to transiently alter signal transduction and protein-protein interactions (PPIs). In this work we try to gain more insight into PPIs and the transient changes in signal transduction in the osmostress pathway of *S. cerevisiae*. The osmostress pathway is composed of two functionally redundant branches, and employs a mitogen activated protein kinase (MAPK) cascade, a central component of stress signalling. We particularly focussed our studies on transient protein-protein interactions occurring between membrane bound proteins and cytoplasmic proteins. Therefore, we developed a new protein interaction assay based on an enzymatic reaction between the two potential interaction partners. This method relies on a mammalian histone methyl transferase (HMT) and a 20 amino acid stretch of its substrate histone H3 protein. This system proved to be an *in vivo* protein interaction assay capable of catching transient changes of interaction rates of signalling proteins.

Using this assay we discovered that the interaction of the membrane protein Sho1 and the MAPKKK Ste11 increases upon osmostress and that this interaction is enhanced in strains incapable of downstream signalling (*Δpbs2, Δhog1)*. Furthermore this interaction depends on Ste50, an adaptor for Ste11, and the osmosensors Msb2 and Hkr1. However, this interaction was found to be independent of the membrane bound adaptor protein Opy2. Additionally, we discovered that the affinity of Sho1 and the MAPKK Pbs2 decreases upon pathway activation, which may reflect the dissociation of the Sho1-Pbs2 complex.

We also investigated how pathway crosstalk is avoided, for the same proteins are shared in different signal transduction pathways. We confirmed that Fus1, a protein necessary for cell fusion during mating, negatively regulates the HOG pathway by interacting with Sho1 and possibly by effecting its expression levels.

Finally, we investigated negative regulatory elements of signal transduction, focussing on the adaptor protein Nbp2, a protein recruiting the phosphatase Ptc1 to the Pbs2/Hog1 complex. We showed that Nbp2 may not exclusively act as a negative regulator for the osmostress signalling cascade, but may also have a positive influence on the HOG pathway, possibly via regulating other MAPK cascades as well.

2 Zusammenfassung

Signaltransduktion ist ein wichtiger zellulärer Prozess, der durch schnelle und exakte Protein-Protein-Interaktionen (PPIs) eine zelluläre Antwort auf verschiedenste Umweltbedingungen, und somit das Überleben der Zelle ermöglicht. In dieser Dissertation versuchten wir mehr über PPIs und im Speziellen über ihre transienten Veränderungen während der Signaltransduktion von Osmostress in *S. cerevisiae* herauszufinden. Der Osmostresspathway besteht aus zwei funktionell redundanten Zweigen und bedient sich einer mitogenaktivierten Proteinkinasenkaskade (MAPK Kaskade), welche auch zentrales Element für weitere Stresssignaltransduktionen ist . Unser spezielles Augenmerk galt hier der Interaktion von Membranproteinen und cytoplasmatischen Proteinen, daher entwickelten wir eine neue Methode, die auf einer enzymatischen Reaktion zwischen zwei potentiellen Interaktionspartnern basiert. Dieser Assay beruht auf einer Histonmethyltransferase (HMT) und seinem Substrat, einem 20 Aminosäuren großen Teil des Histons H3. Dieses System hat sich als ein *in vitro* assay erwiesen, der es ermöglicht, transiente Veränderungen von Proteininteraktionen aufzuzeigen.

Mit Hilfe dieser Methode konnten wir aufzeigen, dass sich unter Osmostress die Interaktion des Membranproteins Sho1 und der MAPKKK Ste11 verstärkt, und dass dieses Signal in Stämmen, die das Osmostresssignal nicht weiterleiten können (*Δpbs2, Δhog1*), weiter verstärkt wird. Außerdem hängt diese Interaktion von Ste50, dem Adaptorprotein von Ste11, und den Osmosensoren Msb2 und Hkr1 ab. Es konnte jedoch kein Einfluss des membrangebundenen Adaptorproteins Opy2 nachgewiesen werden. Weiters entdeckten wir, dass die Affinität von Sho1 und der MAPKK Pbs2 unter Osmostress reduziert ist, was die Dissoziation des Sho1-Pbs2-Komplexes widerspiegeln könnte.

Außerdem untersuchten wir, wie, obwohl die gleichen Proteine von mehreren Signaltransduktionskaskaden gebraucht werden, pathway-crosstalk vermieden werden kann. Wir entdeckten, dass die Expression von Fus1, ein für die Zellfusion benötigtes Protein, den Osmostress-pathway durch die Erniedrigung von Sho1-Proteinlevels negativ beeinflusst.

Letztlich studierten wir negative Regulation der Signaltransduktion mit dem Fokus auf dem Adaptorprotein Nbp2, welches die Phosphatase Ptc1 zum Pbs2/Hog1-Komplex rekrutiert. Wir zeigten, dass Nbp2 nicht nur als negativer Regulator für Osmostresssignaltransduktion dienen könnte, sondern auch einen positiven Effekt, möglicherweise durch die negative Regulation anderer MAPK Kaskaden, auf ihn haben könnte.

3 Introduction

In order to survive and propagate, a cell needs to be able to recognize various environmental conditions and to adapt to them accordingly. To assure this, nature has developed highly intertwined and complex signalling networks that allow cells to specifically react on given signals from their environment. One of the major ways in which cells respond to such environmental signals is to extensively change the pattern of gene expression, leading to increased levels of proteins with required, and to a decrease of proteins with non essential functions. The process from sensing environmental conditions to the induction of adaptation is now commonly termed 'signal transduction', a term that was first used in 1972 by Rensing *et al*. Since then, this topic of research enjoys great popularity in science, for the number of publications per year dealing with signal transduction is still increasing in the new millennium.

Considerable effort has been put into uncovering the mechanisms of signal transduction. However, there is still a lot of work for scientists in this field. Whereas at present sensing mechanisms of environmental conditions as well as the transcriptional changes leading to adaptation to specific conditions are often well-studied, the dynamic processes of protein-protein interaction during the transduction of the signal from sensor to transcription factors is still subject to a lot of speculation. Similar to all eukaryotes, signalling in yeast is mainly carried out by the so-called mitogen activated protein kinase (MAPK) cascades. In the following chapter the components of MAPK signalling cascades will be discussed.

3.1 Architecture of MAPK signalling cascades

3.1.1 The basics modules of MAPK signal transduction

'Kinase' names a protein that adds a phosphate group to a substrate protein. The phosphate group is provided by the hydrolysis of ATP to ADP. Therefore, kinases have an active site that binds ATP and a target protein binding site. Kinases involved in signal transduction have different specificities and are therefore divided into several groups: Histidine kinases, arginine kinases, serine/threonine kinases and tyrosine kinases. However, so called dual specificity kinases exist that can phosphorylate serine, threonine and tyrosine. Histidine and arginine kinases are mainly employed in bacteria, although some are conserved in higher

organisms. On the other hand, serine/threonine kinases and tyrosine kinases are the most common kinases involved in signalling in higher eukaryotes. Mitogen-activated protein kinases (MAPK) belong to the group of serine/threonine kinases phosphorylating SP or TP sites at their substrate proteins. MAPKs regulate various cellular processes in response to extracellular stimuli. Since the focus of this work is on MAPK signal transduction, only those cascades will be described in detail in this introduction (Figure 1)

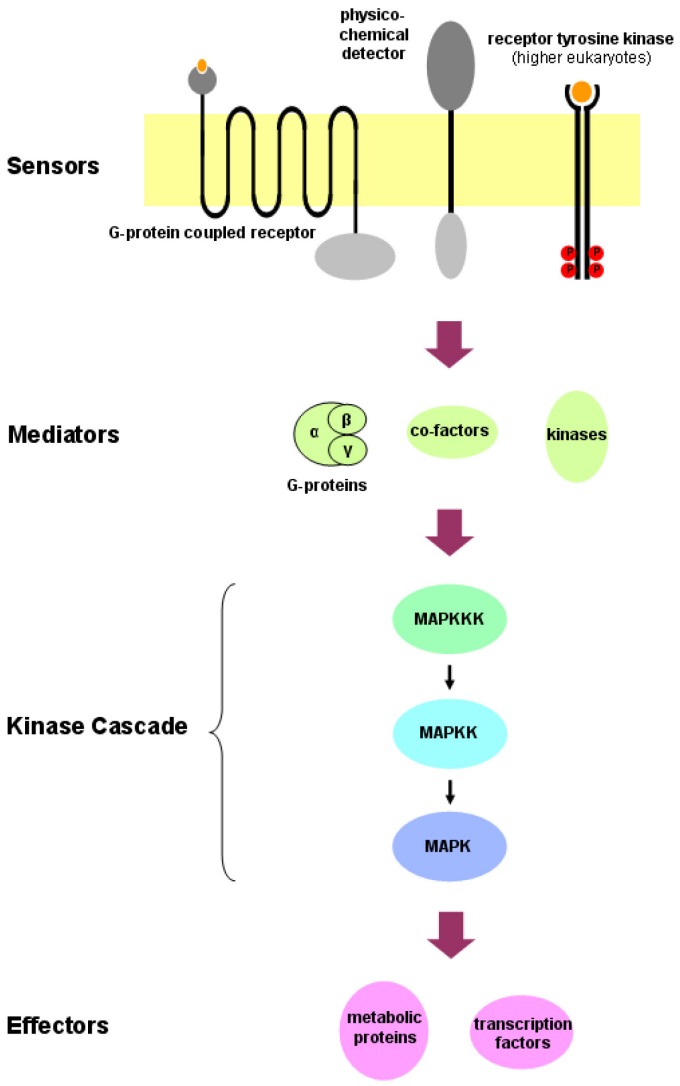


Figure 1: The basic modules of MAPK signalling pathways

The different environmental conditions a cell has to react to are diverse. There are physico-chemical stimuli like heat, cold or changes in the osmolarity, biological conditions like availability of nutrient sources, as well as specific signals like growth factors, pheromone or drugs. Due to the variability of those stimuli, different sensor systems have evolved to obtain necessary information from the environment. Since the plasma membrane is the border

of a cell to its environment, most sensors are membrane bound proteins. Subsequent to stimulus recognition by the sensor protein, an intracellular signal is created, most commonly by a conformational change of the sensor protein itself or by other proteins, found in complex with the sensor. Furthermore, some sensor proteins have kinase-activity and can act at the very first stage of signal transduction. Therefore, they are called receptor tyrosine kinases (RTKs). However, RKTs are only found in higher eukaryotes but not in yeast.

Frequently, the activation of the sensor is followed by the activation of a guanine nucleotide-binding protein (G-protein) previous to the activation of the kinase cascade. G-proteins hydrolyse GTP to GDP and become thereby activated. There are two different kinds of G-proteins: heterotrimeric G-proteins and small G-proteins. The former is composed of three subunits α, β and γ: The α-subunit consists of a GTPase domain. Upon activation, GTP is hydrolysed to GDP, which induces the release of the α-subunit from the β/γ-complex. Further signal transduction can occur via the α-subunit as well as via the β/γ-complex, depending on the activated pathway. The small G-protein has a size of around 20-25kD, which is similar to the size of α-subunit of the heterotrimeric G-protein. Small G-proteins require additional proteins to successfully hydrolyse GTP, for example GTPase accelerating proteins (GAPs) and Guanine nucleotide exchange factors (GEFs). As for heterotrimeric G-proteins, GTP is modified to GDP upon activation. This results in a conformational change inducing further signal transduction via the mitogen activated protein kinase (MAPK) cascade.

Those cascades are usually composed of three kinases, generally named MAPKKK, MAPKK, and MAPK that subsequently phosphorylate its downstream kinase. After the phosphorylation and activation of a MAPK, it generally becomes translocated into the nucleus, activating transcription factors that in turn stimulate the transcription of genes necessary for adaptation.

The transcriptional changes needed for adaptation may range from only little to vast changes in overall gene expression. Furthermore, time is an important factor for adaptation. The response to one environmental condition may need to be fast and transient, another environmental signal may require long-lasting and steady gene expression changes in order to survive and propagate.

3.1.2 Regulation of MAPK-cascades

Since MAPK-cascades govern important processes of cell survival and growth, it is essential for a cell to tightly regulate these signalling pathways. Especially in MAPK signal

transduction, some proteins are shared by the individual pathways. However, a particular signal usually only leads to the activation of the according response. This observation suggests the presence of mechanisms regulating signalling specificity. Moreover, signalling dynamics are of high importance, because a signal can occur in different severities, raising the need of pathway-regulation by flexible response dynamics to decide on the level of response. Lastly, to survive a certain stress condition, it may be necessary for a cell that an active signalling pathway is shut down while the stimulus is still present, as it may be the case after adaptation to a certain condition. As reviewed in Cowan and Storey (2003) there are different strategies to regulate signal transduction and overcome the above mentioned challenges: These strategies involve scaffolding proteins, phosphatases and regulators of protein stability.

A scaffolding protein is a protein that does not necessarily have any catalytic activity itself, but they contain several binding sites for other proteins that are otherwise unable to interact with each other. By binding those proteins, the scaffold protein ensures their adjacency which in turn may lead to the modification of the substrate protein by the catalytically active protein. Scaffold proteins may therefore be involved in specifying a pathway, binding a component that acts in different signalling pathways. Furthermore, only proteins bound to the correct scaffolding protein may be activated by a specific signal.

The challenge of regulating signalling dynamics is mainly tackled by phosphatases and protein turnover and stability: To be able to react swiftly to changing environmental conditions, the turnover of signalling components can be changed upon stimulation of the signalling cascade. Basal levels of MAPK signalling during normal growth conditions can be easily repressed if an essential component of the cascade has a high turnover rate, because such a factor will be degraded before it can be activated. Upon activation of the signalling cascade, the stability of this protein may increase, and therefore allow signal transduction. In contrast, the turnover of a protein involved in MAPK signalling may also increase upon stimulation of the pathway. This allows the cascade to reset faster, if the stimulus is removed or adaptation to the stress is completed. Moreover, phosphatases play an important role in the regulation of signalling dynamics of MAPK cascades. Phosphatases are the antagonists of kinases, their major function in signal transduction being to negatively regulate MAPK cascades in two ways: On one hand, they keep basal activity of signalling cascades during normal growth conditions below a certain threshold that does not induce a cellular response. On the other hand, they negatively regulate active MAPK cascades after the responsible stimulus has ceased or adaptation is complete. Additionally, this negative impact on a MAPK

cascade may result in a positive signal for another pathway, also possibly involving them in governing signalling specificity.

3.1.3 MAPK-cascades and cancer

As mentioned earlier, MAPK signalling is highly conserved in eukaryotes and regulates various important processes within a cell. Therefore, deregulation of these signalling pathways is often fatal and may lead to the development of disease. The perhaps most prominent health condition related to MAPK signalling is cancer development. Therefore studying these signalling processes is crucial for understanding this threat and to be able to develop new treatment strategies. As reviewed in Hanahan *et al*. (2000), cells have to fulfil a number of criteria in order to develop cancer. They have to become independent of growth factors and insensitive to anti-growth factors. They must lose the ability to induce programmed cell death (apoptosis) and overcome senescence, meaning losing the ability to divide. Finally they have to be able to drive angiogenesis and invade tissues for metastasis. Malfunction of MAPK-cascades is jointly responsible for most, if not all of those criteria. To illustrate how MAPK cascades act in cancer formation, the mammalian ERK-pathway will serve as an example.

The ERK pathway regulates diverse processes as cell proliferation, differentiation and survival. It is known to be deregulated in various cancers; therefore it is one of the best studied mammalian MAPK-cascade. It is composed of the MAPKs ERK1 and ERK2, the MAPKKs MEK1 and MEK2, and the MAPKKKs A-Raf, B-Raf, c-Raf-1 and Tpl2 (Roux *et al*., 2004; Dhillon *et al*., 2007). The cascade can be stimulated by a multitude of factors like growth factors, microtubule disorganization and mitogens (Gerits *et al*., 2007). The ERK-pathway is deregulated in about one third of human cancers (Dhillon *et al*., 2007). Most defects of the pathway are found at upstream elements. Still, deregulation also takes place at the level of the transcription factors. Mutations and/or changes in expression levels result in constitutive activation of ERK, which results in constitutive cell proliferation:

Mutations of the G-protein Ras are found in different cancers with different frequencies. The most common mutations happen at codons 12, 13 or 61, resulting in that the G-protein Ras is caught in its active, GTP-bound state, and constitutively binds its effector Raf and other proteins, capable of activating ERK. The b-raf gene was found to be mutated in two thirds of melanomas (Davies *et al*., 2002) with V600E as the most common mutation. This residue is localised in the activation loop and as for the Ras mutants the mutation results in constitutive catalytic activity (Wan *et al*., 2004). Raf-1 mutations that are most commonly

found in acute myeloid leukaemia cells (Zebisch *et al*., 2006), and don't necessarily influence kinase activity, but its non-catalytic functions, which are involved in countering apoptosis (Chen *et al*., 2001, O'Neill *et al*., 2004, Piazolla *et al*.,2005). However, there is still one safety mechanism within cells that has to be deactivated in order to develop cancer: High levels of ERK-signalling can lead to the expression of the proteins p21 and p27. These proteins are CDK-inhibitor proteins, therefore their expression leads to cell cycle arrest. To overcome this safety mechanism, Akt or Rho signalling has to be active in tumour cells. These two pathways are involved in different processes, one of them being cell cycle regulation. Upon activation of these pathways in tumour cells, cell-cycle arrest is overcome and cells can divide unrestrictedly. Furthermore, MAPK phosphatases (MPKs) and Sprouty family members (proteins with a conserved cysteine-rich domain that have antagonistic functions in signalling pathways) were reported to be deregulated in cancer cells (Miyoshi *et al*., 2004; Tsavachidou *et al*., 2004; Bloethner *et al*.,2005; Fong *et al*., 2006) and the list of proteins involved in deregulation of cellular processes grows every day.

3.1.4 Yeast MAPK cascades

Since as described in the chapter above, MAPK signalling is often involved in disease and cancer development, MAPK cascades are an especially important field of study. However, to study stress signalling in higher eukaryotes poses certain challenges. In contrast, *Saccharomyces cerevisiae* is a particularly suitable model organism to study signalling, and especially stress signalling. Many factors and often entire pathways have been found to be conserved between *S. cerevisiae* and higher eukaryotes. Furthermore, being a single cell organism, yeast cells lack the protection that cells of metazoans enjoy, for they do not organise in tissues and are therefore directly exposed to the changes in their environment. Therefore, signalling leading to cell survival can be properly studied in this organism. In this chapter the five MAPK cascades of *S. cerevisiae* will be discussed (Fig. 2). However, it should be noted that other signalling cascades like for example the TOR pathway or cAMP/PKA pathway exist and that they are also important pathways for stress signalling.

Similar to higher eukaryotes, MAPK cascades regulate a variety of important processes within *S. cerevisiae*. The five known MAPK cascades in yeast are (Fig. 2): The spore wall assembly pathway is needed when nutrient conditions force the cell to undergo meiosis and form an ascus, containing four haploid cells. The cell wall integrity pathway is activated if the association of the plasma membrane and the cell wall are altered and prevents

cell lysis. Upon nitrogen or carbon starvation, the filamentous growth pathway is induced leading to changes in cell shape. The pheromone pathway is active during mating of yeast and ensures cell cycle arrest as well as cell fusion. Finally, the high osmolarity glycerol (HOG) – pathway (the pathway mainly studied in this work) is activated upon osmostress, and its activation leads to production and retention of glycerol within a cell. In the following chapters, these five different MAPK cascades of *S. cerevisiae* are introduced.

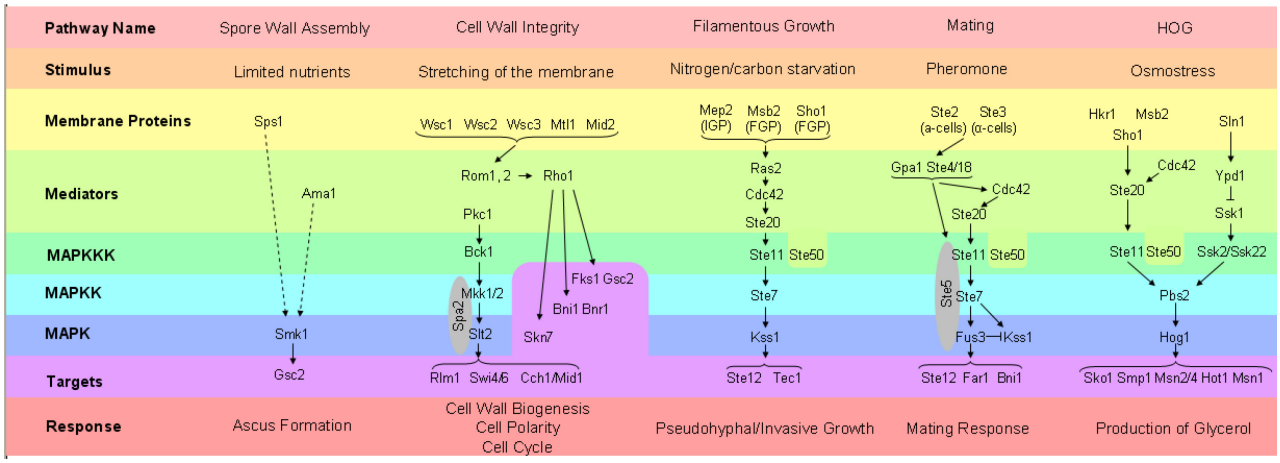


Figure 2: MAPK cascades in yeast

3.1.4.1 Spore Wall Assembly Pathway

When exposed to an environment containing insufficient amounts of nutrients to continue propagation diploid yeast cells undergo meiosis and form an ascus containing four haploid cells. This spore-engulfing layer is composed of four sub-layers: the two inner layers mainly consist of glucan and mannan; the two outer layers are made out of chitin and dityrosine formations (Kupiec *et al.*, 1997, Neiman 2005). Little is known about the signal transduction taking place to enable the formation of this coat. However, Smk1 was identified as a MAPK to be necessary for the assembly of the two outer layers of the spore wall (Krisak *et al.*, 1994). Furthermore, Smk1 was found to be expressed only during sporulation (Pierce *et al.*, 1998). Smk1 interacts with and negatively regulates a subunit of Gsc2, a 1,3-β-glucan synthase that synthesizes the glucan layer and is therefore thought to be necessary to terminate the synthesis of the glucan layer to allow assembly of the chitin layers (Huang *et al.*, 2005).

No MAPKK or MAPKKK have been identified yet, but Smk1 activity was found to depend on Ama1, a meiosis-specific activator of the anaphase promoting complex (McDonald *et al.*, 2005). Another protein important for cell wall formation is the PAK-like

kinase Sps1. *Δsps1* cells were shown to have a phenotype similar to *Δsmk1*. However, Sps1 is not necessary for the activation of Smk1 (Friesen *et al*., 1994). The exact function of these two proteins in the cell wall formation pathway remains elusive.

3.1.4.2 The Cell Wall Integrity Pathway

A well described signalling pathway in yeast is the Cell wall integrity pathway (CWIP), extensively reviewed by Levin *et al*. (2005). This pathway becomes activated upon stretching of the plasma membrane or abnormalities in the connection of the plasma membrane to the cell wall induced by diverse circumstances such as pheromone induced morphogenic changes, hypotonic stress, heat stress, or treatment with various cell wall damaging agents like Zymolyase or Calcofluor White. All these conditions ultimately lead to the activation of the MAPK Slt2 (Mpk1). In case the pathway becomes interrupted by deleting an essential component of the signalling cascade, cells lyse due to the loss of cell wall integrity. Survival for such strains is only possible in medium containing sorbitol or any other supportive osmolyte (Lee *et al*., 1993).

Five transmembrane proteins functioning at the top of this signalling cascade have been described: Wsc1,2 and 3, Mid2 and Mtl1 (Jacoby *et al*., 1998; Verna *et al*., 1997; Rajavel *et al*., 1999). Double knock-out strains of Wsc1 and Mid2 require osmotic support to survive. Both proteins have highly O-mannosylated ectodomains, therefore they are probably acting as mechanosensors (Philip *et al*., 2001), because these modifications are thought to stiffen peptide chains. Wsc1 is localised to sites of polarized cell growth and its deletion is lethal at restrictive temperature. Mid2 deletion mutants fail to survive upon pheromone treatment (Ono *et al*., 1994), because they fail to adapt to mating-induced morphogenic changes. However, unlike Wsc1, Mid2 is evenly distributed at the plasma membrane (Ketela el at., 1999).

Upon activation of the sensors the redundant GEFs Rom1 and Rom2 become recruited to the membrane and nucleotide exchange of the G-protein Rho1 is stimulated (Ozaki *et al*., 1996). Rho1 is localised at sites of polarized cell growth and upon its own activation, a group of effectors becomes in turn activated (Yamochi *et al*., 1994): The kinase Pkc1 (Nonaka *et al*., 1995), the response regulator and transcription factor Skn7 (Alberts *et al*., 1998), the formins Bni1 and Bnr1 (Sagot *et al*., 2002; Evangelista *et al*., 2003) and the cell wall synthesizing enzymes Fks1 and Gsc2 (Drgonova *et al*., 1996; Qadota *et al*., 1996). The MAPK cascade, activated by Pkc1, is composed of the MAPKKK Bck1, the redundant

MAPKKs Mkk1 and Mkk2 and the MAPK Slt2. Mkk1/Mkk2 and Slt2 are bound by the scaffold protein Spa2 (Lee *et al*., 1993; Levin *et al*., 1990; Irie *et al*., 1993; van Drogen *et al*., 200). Slt2 activates the transcription factors Rlm1 and Swi4/6 (Watanabe *et al*., 1997; Madden *et al*., 1997), but also targets the Ca^{2+} channel at the plasma membrane (Cch1/Mid1) (Bonilla *et al*., 2003). When the channel is activated, it allows the uptake of Ca^{2+}, which in turn enhances the transcription of Fks2 and other cell-wall related genes (Bonilla *et al*., 2003). Rlm1 controls the expression of at least 25 genes of which most are involved in cell wall biogenesis (Jung *et al*., 1999). Swi4/6 regulate cell morphogenesis as well as the cell cycle switch from G1 to S. (Breeden *et al*., 2003; Igual *et al*., 1996)

3.1.4.3 The Filamentous Growth Pathway

Yeast as an immobile organism developed the pseudohyphal or filamentous growth pathway to adapt to poor nutrient supply in its direct environment (Robert *et al*., 1994). Upon pathway induction, major cytoskeleton rearrangements stretch the cell, accompanied by changes in the budding pattern: In the pseudohyphal growth of diploid cells, budding occurs bipolar, creating daughter cells at the opposite cell poles. In the invasive growth of haploid cells axial budding takes place, the daughter cell is budded from the same cell pole, which is adjacent to the birth end of the mother cell. This directed budding combined with the new shape of the cells is an excellent mechanism to allow efficient spreading of the colony and access possible nutrient sources. Although the diploid pseudohyphal growth pathway (PGP) and the haploid invasive growth pathway (IGP) are not identical (e.g. nitrogen starvation induces the PGP whereas glucose starvation induces the IGP) they share components and mechanisms of MAPK signal transduction.

The common phosphorylation cascade is composed of the PAK like kinase Ste20, the MAPKKK Ste11, the MAPKK Ste7 and the MAPK Kss1. Ste20 activity needs the active G-protein Cdc42, which depends on active Ras2, a small GTP binding protein. Ras2 in turn needs stimulation by the GEF Cdc25. The dynamics of those processes and the exact functions of proteins involved in FG signalling still have to be uncovered. For pathway induction of the haploid IGP the membrane and putative sensor proteins Sho1 and Msb2 are required (Cullen *et al*., 2004), for the diploid PGP the high affinity ammonia permease Mep2 that acts as nitrogen sensor is essential (Lorenz *et al*., 1998). However, the need for Mep2 can be bypassed by dominant active Ras2 mutants, indicating that Mep2 is only needed for activation of Ras2, but is not involved in signalling processes downstream of Ras2. Under

normal conditions, the MAPK Kss1 acts as a repressor by binding to the transcription factor Ste12 (Bardwell *et al*., 1998). Activation of Kss1 via the FG MAPK cascade leads to the dissociation and activation of Ste12 and the transcription factors Tec1. Ste12 and Tec1 enable transcription of proteins necessary for filamentous growth, as for example *PGU1* (pectolytic enzyme), *MUC1* (cell surface glycoprotein, flocculin) and *CLN1* (G1 cyclin) (Madhani *et al*., 1997; Madhani *et al*., 1999)

Ras2 does not only activate the described MAPK cascade, but it also activates the cAMP dependent protein kinase pathway. Upon Ras2-dependent stimulation of the adenylat cyclase Cyr1, cAMP levels within the cell rise and Bcy1, the regulatory subunit of the PKA is tethered to cAMP thereby releasing and activating the partially redundant kinases Tpk1, Tpk2 and Tpk3. They in turn target transcription factors as for example Msn2/4 (Gorner *et al*., 2998), but also metabolic enzymes or other kinases.

3.1.4.4 The Pheromone Pathway

Yeast can exist and propagate as haploid and diploid organism. Haploid cells exist as two mating types, a or α. To find a cell of the opposing mating type, a-cells secrete a-factor and express α-factor-receptors at the plasma membrane, whereas α-cells secrete α-factor and express a-factor receptors. The process of uniting two haploid cells to one diploid cell is called mating and is regulated by a MAPK cascade (reviewed in Chen *et al*., 2007; Bardwell 2005, see figure 3B). For those actions, profound changes within the cells have to take place: Cells of different mating type have to identify each other and a so called shmoo, a cellular bulge that grows in the direction of the other cell, has to be formed. Furthermore, the cell cycle has to be arrested and controlled plasmogamy and karyogamy have to be ensured.

Upon binding of a- or α- factor to their respective receptor (Ste2 for α- and Ste3 for a-factor) the MAPK cascade becomes activated (Burkholder *et al*., 1985). Ste2 and Ste3 are G-protein coupled receptors, which activate a heterotrimeric G-protein composed of Gpa1 (α-subunit), Ste4 (β-subunit) and Ste18 (γ-subunit) (Blumer *et al*., 1990). This activation results in two events: The PAK-like kinase Ste20 becomes activated by the now active Cdc42 (Lamson *et al*., 2002), and the scaffold protein Ste5 is recruited by the Ste4/Ste18 complex (Pryciak *et al*., 1998). The recruitment of Ste5 to the plasma membrane allows the activation of the cytoplasmic MAPKKK Ste11 by the membrane associated Ste20. Ste11 now activates the MAPKK Ste7 which in turn activates the MAPK Fus3 by subsequent phosphorylation. The main scaffolding function of this pathway is held by Ste5, which binds Ste11, Ste7 and

Fus3 and is thought to therefore specify the pheromone induced signal (Drogen *et al.*, 2000). Fus3 phosphorylates the transcriptional activator Ste12, which induces the expression of mating genes, like for example Fus1, a protein required for cell fusion. Additionally, Fus3 phosphorylates Far1, which mediates cell cycle arrest in G1 that is necessary to ensure proper mating (Elion *et al.*, 1993). Another important protein is Bni1, which also becomes phosphorylated by Fus3; it is involved in polarized growth of the shmoo (Matheos *et al.*, 2004). Another function of Fus3 is the inhibition of the filamentous growth pathway: As mentioned before, activated Ste7 also leads to activation of Ste12 via the MAPK Kss1, which potentially activates genes for the filamentous growth pathway. How mis-signalling is avoided will be described in chapter 3.1.4.6.

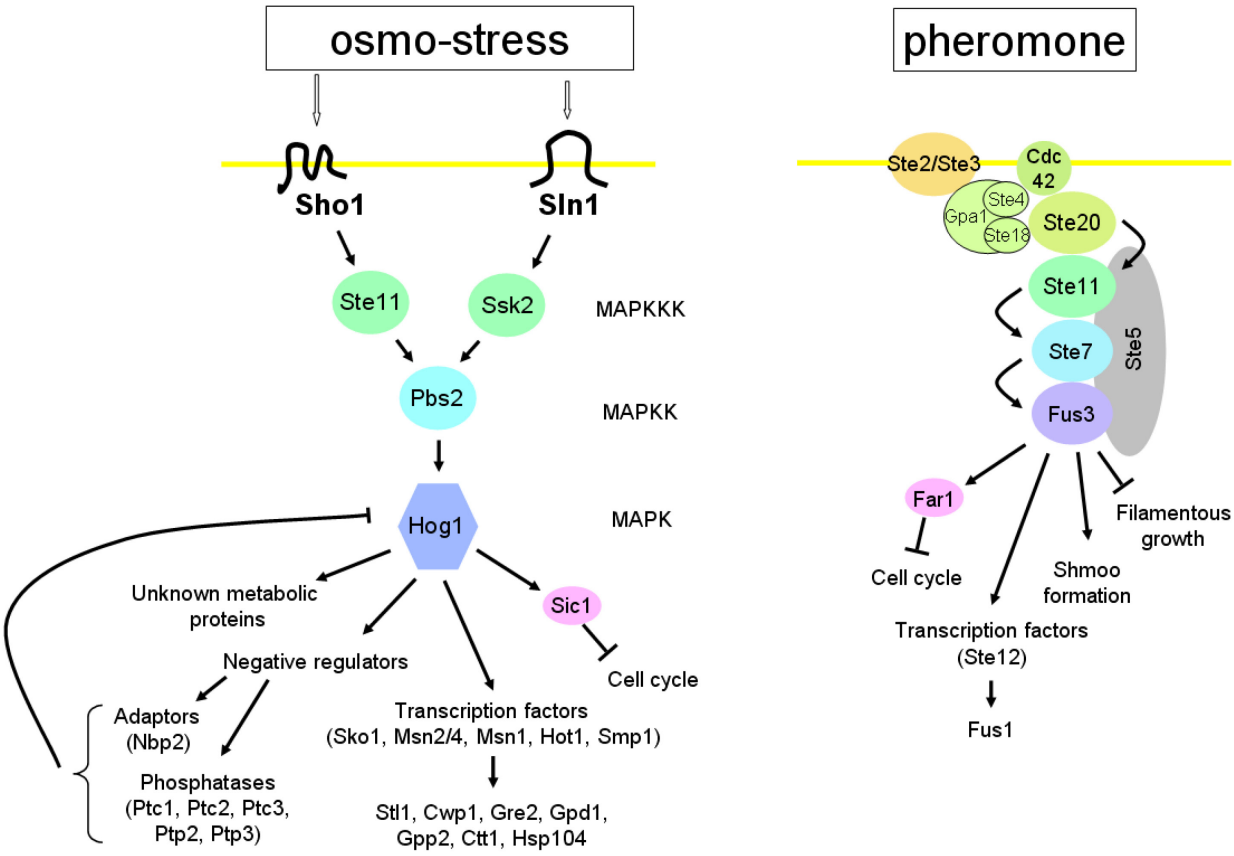


Figure 3: Two MAPK pathways in yeast. A The osmostress pathway. **B** The pheromone pathway. See text for explanation

3.1.4.5 The High Osmolarity Glycerol Pathway:

The HOG pathway was originally discovered to be induced upon osmotic stress. The MAPK Hog1, from which the pathway derived its name, is a stress activated protein kinase (SAPK) that is highly homologous to the mammalian MAPK p38. p38 is involved in stress response,

of higher eukaryotes but it also controls various other processes as for example cell survival and death, differentiation, growth and the immune response (Zarubin *et al*., 2005; Gerits *et al*., 2007). Lately it was shown that the yeast HOG pathway is not only activated by osmotic stress, but also upon other stimuli like cold stress (Hayashi *et al*., 2006), citric acid stress (Lawrence *et al*., 2004) and oxidative stress (Bilsland *et al*., 2004), indicating its function as a true SAPK. Besides the different ways to activate the yeast HOG pathway, this chapter focuses only on facts concerning osmostress.

The dehydration of a yeast cell upon osmostress results mainly in two things: cell shrinkage and turgor loss. Dehydration upon osmotic challenge is a fast process, completed in about one minute. To survive this condition, the cell needs to regain its turgor and cell volume and has to overcome the growth arrest. This adaptation is a two step process: First, the immediate survival has to be assured by limiting the efflux of water. This is accomplished by the retention of the competitive osmolyte glycerol. This process is controlled by the membrane channel Fps1 that is involved in glycerol transport. However, whereas under normal growth conditions its basal activity depends on Hog1, it was shown to become shut in a Hog1 independent way during osmostress (Tamas *et al*., 1999). Second, the cellular metabolism machinery has to undergo changes that allow the cell to regain homeostasis by the production of glycerol which in turn also leads to incorporation of water back into the cell (Brown *et al*., 1978). Hog1 governs those events and fulfils dual function in adaptation to salt stress: On one hand, it alters transcription and induces the expression of genes necessary for survival. On the other hand, Hog1 directly targets metabolic proteins important for adaptation.

Hog1 was first discovered to be necessary for glycerol accumulation by Brewster *et al*. (1993). *Δhog1* mutants were viable on YPD medium; however they couldn't survive when shifted to YPD containing 0,9M NaCl. Hog1 is a protein of 435 amino acids, containing a highly conserved kinase domain at its N-terminus. Upon osmostress, Hog 1 becomes phosphorylated and activated by the MAPKK Pbs2 at Thr174 and Tyr176. While localised evenly in the cytoplasm during vegetative growth, the activation of Hog1 leads to its nuclear import in an Nmd5 dependent manner (Ferrigno *et al*. 1998). Hog1 induces osmostress dependent gene expression, which does not affect the cell until about 30 minutes after induction (Albertyn *et al*., 1994). Around ten percent of all genes can be expected to have altered gene expression upon osmotic stress. Large scale studies have been performed to analyse gene expression during salt stress which gave further insight into the matter of osmoadaptation (Rep *et al*. 2000, Posas *et al*. 2000, Gasch *et al*. 2000, Causton *et al* 2001,

Yale and Bohnert 200, reviewed in Hohmann 2002). These studies revealed that the cluster of genes that is exclusively altered in expression levels by osmostress is rather small. Most genes are also up- or downregulated by other stresses. Those genes are part of the general stress response and encode for proteins that generally act under conditions that challenge cell survival or proliferation. These proteins are involved in diverse processes, for example gene expression, protein production, protection from oxidative damage and protein denaturation, and redox and carbohydrate metabolism.

It has been shown that the transcriptional response to osmotic stress is mainly a transient event. Hog1 governs those transcriptional changes by activating six different transcription factors:

The first transcription factor targeted by Hog1 is Smp1. Smp1 was shown to interact with and be phosphorylated by Hog1 (De Nadal *et al*., 2003). It was shown to regulate the transcriptional activity of transcription of osmostress genes like *STL1* (Glycerol transporter) and *CWP1* (cell wall mannoprotein).

Another transcription factor targeted by Hog1 is Sko1, a protein containing a bZIP motif that belongs to the CREB (cAMP response element binding) family. Under normal conditions, Sko1 recruits the general transcriptional repressor complex Ssn6/Tup1. Active Hog1 phosphorylates Sko1 at Ser 108, Thr113, and Ser126 (Proft *et al*., 2001), reducing its affinity towards Ssn6/Tup1, which in turn allows transcription of the former repressed genes. Furthermore, it has been shown that activated Sko1 also acts as an activator that recruits the SAGA histone acetylase and the SWI/SNF nucleosome-remodeling complex (Proft *et al*., 2002). Sko1 controls the transcription of genes that ensure the export of sodium (*ENA1*, *HAL1*) (Gaxiola R, *et al*. 1992; Pascual-Ahuir A, *et al*. 2001) or the metabolic gene *GRE2* (Rep *et al*., 2001).

Besides Hog1's function in activating or deactivating transcription factors by phosphorylation, Hog1 is also directly involved in transcriptional activation of stress genes. It was shown that active Hog1 becomes tethered to the promoter regions of target genes, and recruits a histone deacetylase complex Rpd3/Sin3 to the chromatin, inducing transcription of stress-related genes (de Nadal *et al*., 2004). Interestingly, although histone deacetylation is generally known as a repressor of gene expression, deletion of Rpd3 resulted in a decrease of transcription, indicating that it acts as an activator. Additionally, Hog1 mediates recruitment of RNA polymerase II to the promoter to activate stress-dependent transcription (Alepuz *et al*., 2001 Alepuz *et al*., 2003). The transcription factors involved in this process are discussed in the following paragraph.

Hot1 is a transcription factor that is phosphorylated by Hog1; however, this phosphorylation does not change its activity. Hot1 is localised in the nucleus under normal and stress conditions. During stress, Hot1 tethers Hog1 to the promoter, which in turn mediates recruitment of RNA polymerase II to the promoter to activate stress-dependent transcription (Alepuz *et al*., 2003). Hot1 controls the expression of Gpd1 and Gpp2, two main enzymes of glycerol biosynthesis (Rep *et al*., 1999). Furthermore, it transiently induces *STL1* expression, a glycerol-proton-symporter of the plasma membrane (Ferreira *et al*., 2005).

Msn2 and Msn4 are partially redundant transcription factors that mediate general stress response, including osmostress response. Upon different stress conditions, Msn2 and Msn4 become translocated into the nucleus, however, to date, no Hog1 dependent phosphorylation could be observed and the translocation is independent of Hog1 (W. Reiter, unpublished data). In the nucleus, Msn2 and Msn4 bind DNA at the STRE elements in the promoter regions of stress induced genes via their Zn-finger motifs (Martinez-Pastor MT, *et al*. 1996). Similar to Hot1, Msn2 and Msn4 are also thought to tether Hog1 to osmostress regulated promoters, thereby recruiting the PolII complex (Alepuz *et al*., 2001). Genes regulated by Msn2/4 are for example the metabolic gene *GRE2*, the catalase encoding *CTT1* or the chaperone encoding gene *HSP104*.

Yet another target of Hog1 is Msn1. Its C-terminus coding for a DNA binding domain was shown to be structurally related to Hot1. Msn1 regulates the expression of genes mainly controlled by STRE elements and its deletion results in decreased expression levels of *GPD1*, *GPP2*, *CTT1* and *STL1* (Rep *et al*., 1999).

Despite many reports describing Hog1 function in the nucleus, it was recently reported that the import into the nucleus is not crucial for immediate survival of the cell during osmotic challenge. Mutants lacking Nmd5, a karyopherin which is necessary for Hog1 translocation are still able to grow on YPD containing 1M sorbitol (Westfall *et al*., 2008). This finding is coherent with the fact that osmotolerance can not solely depend on the rather slow transcriptional activation of respective genes. The exact non-transcriptional functions of Hog1 remain elusive. However, Hog1 may primarily not be important for gene expression, but for the establishment of the metabolic conditions for elevated glycerol production and retention and for the immediate retention of glycerol. Unpublished experiments of our lab showed that around 65 proteins that are no transcription factors become phosphorylated in a Hog1 dependent manner during osmostress (W. Reiter, J. Veis, I. Dohnal unpublished data). The identified proteins have various functions, however, some clusters, as for examples

vesicle formation and transport and the regulation of endocytosis, can be identified (W. Reiter, personal communications).

Upon equilibration of the cell to the environment, activated Hog1 needs to be dephosphorylated, because constitutively active Hog1 is lethal. Phosphatases targeting Hog1 include Ptc1, Ptc2, Ptc3, Ptp2, and Ptp3 (Mattison *et al*., 2000; Warmka *et al*. 2001, Ferrigno *et al*. 1998). Whereas Ptc1 is thought to keep basal Hog1 phosphorylation low, the other phosphatases function partially redundantly on turning off the HOG pathway after adaptation occurred. Dephosphorylation of Hog1 results in its export from the nucleus in a Crm1 dependent manner.

The two branches of the HOG pathway:

Two individual branches regulate the activity of Hog1, the SHO1 branch and the SLN1 branch (Fig. 4). Disabling one of the two branches still allows sufficient Hog1 activation to acquire a competent stress response. However, the two pathways are not completely redundant: The SHO1 branch needs a minimum of 0,3 M NaCl to induce phosphorylation of Hog1, whereas signalling via the SLN1 branch is possible at concentrations as low as 0,1 M (Maeda *et al*., 1995). Furthermore, SLN1 seems to have a linear dose response up to 0,6M NaCl, whereas the SHO1 branch sensor seems to initiate a switch like response. It was shown by van Wuytswinkel *et al*. (2000) that the signalling efficiency of the SHO1 branch is heavily reduced at concentrations above 1,4 M NaCl. Another striking, yet unexplained difference between those two branches is that while Sln1 is localized evenly on the cell membrane, the Sho1 sensing complex is localised to places of polarised cell growth.

Both branches have their specific MAPKKKs, (Ste11 for the SHO1 branch and Ssk2 and Ssk22 for the SLN1 branch); however, input signals of both lead to the activation of one MAPKK Pbs2. In the first publication of Pbs2, the striking similarity to Ste7, the MAPKK of the mating pathway, was already described (Boguslawski *et al*., 1987). Pbs2 is a protein of 668 amino acids, containing a highly conserved kinase domain at the C-terminus. Wild type Pbs2 is distributed evenly throughout the cell. However, a catalytically inactive mutant was shown to be localized to the bud neck or bud tip upon osmostress (Reiser *et al*., 2000). As mentioned above, Pbs2 is phosphorylated by either one of the MAPKKKs Ssk2/Ssk22 or Ste11 upon osmotic stress, which in turn results in phosphorylation of Hog1 by Pbs2. Pbs2 activation via Ste11 is dependent on the membrane protein Sho1. They interact with each other specifically via a proline rich region in Pbs2 and the SH3 domain of Sho1 (Tatebayashi

K, *et al*., 2003). Furthermore, Pbs2 is thought to have important scaffolding functions for the SHO1 branch of the HOG pathway, by bringing together Sho1, Ste11 and Hog1 (Posas *et al*., 1997).

The SLN1 branch

The name-giving, sensor histidine kinase Sln1 stands at the top of the SLN1 branch and is a negative regulator of this signalling pathway: A null mutation results in lethality due to the hyperactivation of Hog1 (Janiak-Spens *et al*., 1999). Sln1 consists of two transmembrane domains, a histidine kinase domain and a receiver domain (Ota *et al*., 1993). Being evenly distributed on the cell membrane, Sln1 was shown to cluster upon osmostress. Treatment with nystatin, a drug that induces cell shrinkage and thus mimicking osmostress, activates the SLN1 branch, indicating that Sln1 is sensing turgor pressure (Reiser *et al*., 2003). The branch is composed of a phosphorelay system, similar to bacterial two component systems: Under normal conditions, the histidine kinase of Sln1 is active, and phosphorylates a response regulator domain at its own C terminus. Subsequently, the response regulator Ypd1 becomes phosphorylated by Sln1, and in turn Ssk1, a second response regulator, is phosphorylated and thereby inactivated. Upon hyperosmotic shock, Sln1's histidine kinase activity decreases, leading to accumulation of unphosphorylated Ssk1. Unphosphorylated Ssk1 can bind to the redundant MAPKKKs Ssk2/Ssk22 and induces their autophosphorylation and therefore activation (Posas *et al*., 1998) which in turn results in the phosphorylation of the MAPKK Pbs2.

The SHO1 branch

The SHO1 branch is the more complex and until now the more elusive branch of the HOG pathway: Several components are not unique to this signalling cascade; therefore, the question of how signalling specificity is guaranteed is a yet not sufficiently answered question in this field of research. A scheme of the components involved in the signalling of the SHO1 branch is depicted in fig. 4A. The different members of this signalling pathway are introduced below:

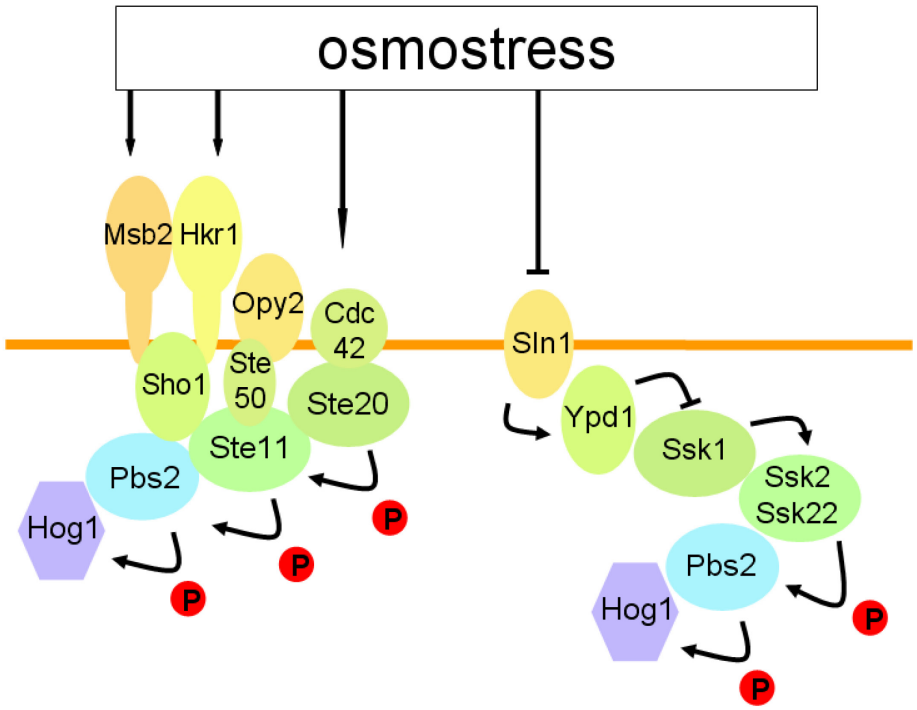


Figure 4: The two branches of the HOG pathway. See text for explanation

Transmembrane proteins Msb2, Hkr1, Sho1 and Opy2

The transmembrane proteins Msb2 and Hkr1 are both members of the mucin family. Mucins are highly glycosylated and are therefore highly hygroscopic proteins. This feature makes them very plausible candidates for osmosensing, because changes of osmolytic parameters and therefore the availability of water molecules may expand or shrink the mucin domain substantially, which potentially leads to conformational changes to their active form. Both, Msb2 and Hkr1 have a large, extracellular, C-terminal domain containing the mucin motif and a transmembrane domain. Msb2 has been shown to interact with Sho1 and the small G-protein Cdc42. Furthermore, Msb2 has been recently describes as an osmosensor of the SHO1 branch, cooperating with Hkr1 to convert high osmolarity stimuli into an intracellular signal (Cullen *et al*. 2004; Tatebayashi *et al*., 2007).

Sho1 has previously been described as a putative osmosensor able to directly activate the MAPKK Pbs2 upon osmostress in an SLN1 branch independent manner (Maeda *et al* 1995). However, as mentioned above, recent evidence suggested that Msb2 and Hkr1 are the actual osmosensors, with Sho1 acting at the same level or rather downstream of them. Sho1 is composed of four hydrophobic transmembrane domains at the N-terminus, and an SH3 domain at the C-terminus. SH3 domains are protein interaction domains that bind to proline-

rich sequences containing a core PXXP motif (Sparks *et al*., 1994). Sho1 is essential for the SHO1 branch because a *Δsho1* mutant is osmosensitive in an SLN1 branch deficient strain. Furthermore, it has been postulated that it is a major role of Sho1 is to localise the MAPKK Pbs2 to the plasma membrane, therefore bringing it into the special vicinity of the MAPKKK Ste11. Both, Msb2 and Sho1 play not only a role in the HOG pathway, but also in the filamentous growth pathway (Cullen *et al*., 2004). However, pathway specific interactions and mechanisms are yet to be determined.

Another transmembrane protein that was recently discovered to have an impact on the SHO1 branch is Opy2. It is highly glycosylated and has one single transmembrane domain. It is thought to interact with Ste50 and thereby recruiting it together with Ste11 to the plasma membrane (Wu, *et al*. 2006, Ekiel *et al*., 2009) (Ste11 and Ste50 will be described in one of the following paragraphs, page 24). *Δssk1Δopy2* strains are osmosensitive, therefore Opy2 is essential for the SHO1 branch. An indication that Opy2 may be important to distinguish the HOG and the pheromone pathway is that it was first described as an overexpression mutant that was able to overcome cell cycle arrest induced by pheromone (Edwards *et al*. 1997). This finding suggests that that the high abundance of Opy2 may redirect an incoming pheromone signal away from its correct response, possibly leading to Hog1 phosphorylation instead.

Cdc42

Cdc42 is an essential gene encoding a small Ras-like GTPase. During normal growth conditions it is necessary for establishing cell polarity and reorganizing the cytoskeleton (Johnson *et al*., 1990; Adams *et al*., 1990). Moreover, Cdc42 has been found to play a role in the HOG pathway, as well as in the FG and the mating pathway (Raitt *et al*., 2000; Reiser *et al*., 2000; Peter *et al*., 1996). It is localised at the plasma membrane at sites of polarised cell growth (Ziman M, *et al*., 1993). Lacking any transmembrane domains, Cdc42 has a prenylation site at the C-terminus for membrane anchorage. Cdc42 is associated with the three GTPase activating proteins (GAPs) Bem3, Rga1, and Rga2 and its guanine nucleotide exchange factor (GEF) Cdc24, which is necessary to exchange GDP with GTP after successful hydrolysis (Madden *et al*., 1998). Cdc42 is thought to act as the mediator between the sensor complex and the kinase cascade that starts with the activation of the PAK-like kinase Ste20 in a Cdc42 dependent manner (Leberer *et al*., 1997; Raitt *et al*., 2000) However, to date it is not known, how Cdc42 becomes activated.

The kinases Ste20 and Ste11 and the adaptor protein Ste50

Ste20 is a member of the p21-activated kinase (PAK) family (Dan *et al*., 2001). It is composed of an N-terminal Cdc42- Rac Interactive Binding (CRIB) domain, a PAK-box domain, and a C-terminal kinase domain. During signal transduction, Ste20 becomes activated by Cdc42 and targeted to the membrane at sites of polarised cell growth (Leberer *et al*., 1997). Thereby, the autoinhibitory function of the CRIB domain is inactivated, and the kinase is activated (Ash *et al*., 2003; Lamson *et al*., 2002), which in the case of osmostress leads to autophosphorylation of Ste20 (Leberer *et al*., 1997) and subsequent phosphorylation of Ste11 (Wu *et al*., 1995). The function of Ste20 can partially be compensated by Cla4, another member of the PAK family. Therefore, *Δste20* mutants in a SLN1-deficient strain are only slightly osmosensitive (Cvrckova, *et al*., 1995). However, this phenotype can be complemented by overexpression of Cla4 (Raitt *et al*., 2000). Beside its function in the HOG pathway, Ste20 is also the activator or Ste11 in the FG and mating pathway and it was first described to be necessary for the transmission of pheromone signal from the G-protein to downstream elements (Leberer *et al*. 1992). Furthermore, Ste20 was also shown to play a role in chromatin condensation during H_2O_2 induced cell death, a process that resembles mammalian apoptosis. It was shown that upon H_2O_2 treatment of cells, Ste20 is imported to the nucleus and phosphorylates serine 10 of histone 2B, a modification that is necessary for chromatin condensation during cell death (Ahn *et al*., 2005).

Ste11 is the MAPKKK of the mating-, osmostress- and filamentous growth response of *S. cerevisiae* (Roberts *et al*., 1994; Raitt *et al*., 2000; Reiser *et al* 2000; Peter *et al*., 1996). It is a protein of 717 amino acids, and is composed of 3 different domains: The Sterile Alpha Motive (SAM) domain at the N-terminus, that interacts with the adaptor protein Ste50, a central autoinhibitory domain that binds and inhibits the catalytic activity of the C-terminus during normal growth conditions, and the MAPKKK domain at the C-terminus. Ste11 was first described as a temperature sensitive mutant that is insensitive to cell cycle arrest by α-factor (Hartwell, 1980) but was shown to be phosphorylated and activated by Ste20 during osmostress, pheromone treatment or nutrient starvation (Wu, *et al*., 1995). To accomplish that, Ste11 and Ste20 have to be in close vicinity. This is ensured by recruiting Ste11 to the plasma membrane. During osmostress, a number of specific proteins play a role in this recruitment process: Cdc42, Opy2, Sho1 and Ste50 (Wu *et al*. 2006; Truckses *et al*., 2006, Kwan, *et al*., 2006; Westfall PJ, *et al*. 2004). After correct localisation and activation of Ste11, Pbs2 in turn becomes phosphorylated and activated by it. Additionally, upon

activation, Ste11 increases its turnover rate by a ubiquitin-dependent protein degradation mechanism (Esch *et al*., 2002).

Ste50 was first reported to lead to supersensitivity to alpha factor when overexpressed (Rad *et al*., 1992). It is composed of an N-terminal SAM domain, which interacts with Ste11, and a C-terminal Ras associated (RA) domain. Ste50 is involved in osmostress-, FG- and pheromone response. It serves as an adaptor protein for Ste11, which it regulates in various ways: During osmostress and in the FG pathway it is involved in the recruitment of Ste11 to the membrane, it inhibits the C-terminus of Ste11 to bind its N-terminus, and modulates Ste11 autophosphorylation (Wu *et al*., 1999). Furthermore, it was shown to link Ste11 with the Cdc42/Ste20 complex to allow downstream signalling (Truckses *et al*. 2006). Although, like Ste11, Ste50 is acting in three different pathways, it may contribute in some way to signalling specificity, because while Ste50 is essential for the SHO1 branch and the FG response, it is of importance, but not essential for full activation of the pheromone response.

Nbp2 - a negative regulator of the HOG pathway

Nbp2 is part of the machinery that inactivates Hog1 during osmostress adaptation (Mapes *et al*., 2004) by recruiting the phosphatase Ptc1 to the Pbs2-Hog1 complex. Nbp2 is an SH3 domain containing, cytoplasmic protein of 236 amino acids. Furthermore, it was shown that Nbp2 interacts with Ste20 (Winters *et al*., 2005) and is involved not only in Hog1 downregulation but also in the CWI pathway (Ohinkuni *et al*., 2003; Garcia *et al*., 2009). Therefore, Nbp2 may also be a factor contributing to sustained signalling specificity, however the exact mechanisms for that remain elusive.

Rtn2 – a novel component of the SHO1 branch

Rtn2 is a member of the reticulon protein family. Reticulons are an evolutionary conserved protein family that have been mainly associated with the endoplasmic reticulum (ER). Both yeast Rtn proteins (Rtn1 and Rtn2) were proposed to shape the ER by only stretching through one of the two layers of its membrane, therefore bending the bilayer: Deletion of Rtn1 and Rtn2 was shown to result in the conversion of peripheral ER to membrane (Voeltz *et al*., 2006). Another publication describes that ER membrane-bending proteins are necessary for the formation of nuclear pores (Dawson *et al*., 2009). Moreover, reticulons were also shown to be of importance for trafficking and vesicle formation (reviewed in Yang *et al*., 2007).

Interestingly, Rtn2 was identified to additionally contribute to SHO1 branch signalling by a genetic screen performed in our lab by S. Salah and V. Reiser (unpublished data). An EMS mutagenesis was performed in an SLN1 branch deficient strain, and mutants that were defective in Hog1 signalling, but still had a functional mating response were chosen for further analysis. *Δrtn2 Δssk1* double deletion strains were found to be salt sensitive, indicating that Rtn2 is necessary for full activation of an osmoresponse via the SHO1 branch.

3.1.4.6 How to guarantee signal specificity in yeast MAPK pathways

As it has been pointed out several times in the introduction of this thesis, some proteins are employed by more than one MAPK pathway in budding yeast. However, a defined stress condition leads to the activation of a corresponding cellular response, circumventing erroneous signalling (Fig. 5). Here, an example will be given that illustrates how signalling specificity may be assured in yeast.

The MAPKKK Ste11 is part of three different MAPK cascades: The pheromone-, the osmostress and the filamentous growth pathway. Yet, all three different signalling cascades are activated by different stimuli and induce the respective response. In the case of Ste11 signalling specificity is guaranteed by different scaffold proteins. During osmostress signalling, the MAPKK Pbs2 acts as scaffold protein, binding Ste11 and Hog1 and therefore forcing the cascade into an osmostress response (Posas *et al*., 1997). In the pheromone pathway, the scaffold protein Ste5 is essential for signalling specificity. It tethers the MAPKKK Ste11, the MAPKK Ste7 and the MAPK Fus3 in one complex, therefore forcing Ste11 to activate the bound MAPKK Ste7 and induce a pheromone response (Marcus *et al*., 1994). In contrast to the HOG and the pheromone pathway, there is no known scaffolding factor employed in the FG pathway. Therefore, the absence of both, Pbs2 and Ste5 in the signalling complex during FG signalling is thought to lead to signal specificity. However, the MAPKK for the pheromone and the FG pathway is Ste7 that can activate both MAPKs, Kss1 and Fus3 (Schaeffer *et al*., 1999). In both signalling cascades, Kss1 becomes activated, however, during pheromone response, Fus3 is the dominant MAPK during pheromone signalling, repressing Kss1-specific signal transduction in the following way: To avoid mis-signalling during pheromone response, Fus3 phosphorylates Tec1, a transcription factor that is associated with Ste12. Tec1 is required for filamentous growth gene expression, and is ubiquitinated and degraded upon phosphorylation during pheromone response (Chou *et al*., 2004; (Bao *et al*., 2004).

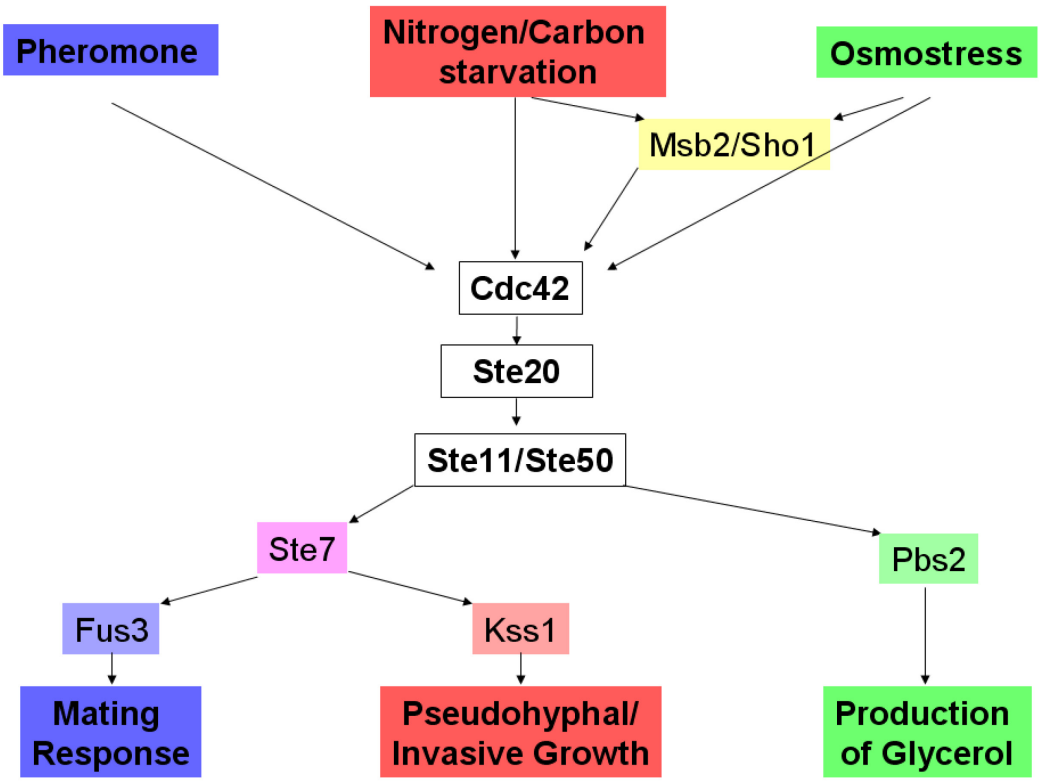


Figure 5: The question of signalling specificity. Cdc42, Ste20, Ste11 and Ste50 are employed by three different MAPK pathways.

3.2 Methods to study signal transduction

Protein-protein interactions (PPIs) and signal transduction control essential processes in all living organisms and have been associated to disease and cancer development. Therefore, much effort has been put into the investigation of PPIs by the scientific community, revealing more than 20.000 PPIs just in yeast (Stelzl *et al*., 2006). To examine the complex protein network within a cell, various approaches have been developed to research this topic. Whereas early approaches to investigate PPIs are based on genetics and biochemistry, the recent assays rely on fluorescence and live-cell imaging. This chapter introduces the most commonly used classic and recently developed methods.

3.2.1 Yeast two hybrid (Y2H)

In 1989 Fields and Song developed the Y2H as a novel, genetic approach, to ascertain whether two proteins can possibly interact. Since then this assay was not only used for researching specific interactions, but also for large scale studies. Two genes encoding for the protein of interest (X and Y) are fused to a DNA binding domain (DBD, bait) and a transcription activation domain (AD, prey) respectively, and are transformed into yeast cells of the opposite mating type. Cells are mated, and therefore diploid cells containing both plasmids are created. Upon interaction of protein X and Y, the DBD and AD are brought into close vicinity, and their function as a transcription factor is restored. As a result, a reporter gene is expressed. The *LacZ* gene is often used as a reporter, allowing not only qualitative assays but also the acquisition of quantitative data of protein interactions by measuring the β-galactosidase activity of the reporter. The advantages of this method are the simplicity of the system, and the possibility to screen large amounts of candidate proteins. The disadvantages are that overexpression is required, and that the localisation of protein is forced to the nucleus, which may distort endogenous conditions and therefore the results of the experiment. Furthermore, membrane proteins are excluded from this assay because of the same reason. If non-yeast proteins are tested by Y2H, there is an additional risk of false positives or negatives due to of wrong post-translational modifications, or simply non-native interactions, because proteins find themselves in an artificial protein context.

3.2.2 Co-immunoprecipitation (CO-IP)

Immunoprecipitation (IP) is a method of precipitating a specific protein from a whole cell extract using antibodies. Co-IP precipitates the protein with all its binding partners it forms a complex with. This is usually performed with specific antibodies immobilized on an insoluble matrix (usually agarose beads). Upon incubation of those beads with the whole cell extract, the specific protein together with its binding partners is captured. After several washing steps, the proteins are eluted and further analyzed by western analysis or mass spectrometry (MS). The advantage of Co-IP is that the results are more reliable than from Y2H, and new interaction partners may be identified if mass spectrometry is performed consequently. Proteins don't necessarily need to be overexpressed, and the experiment can be performed in any organism of interest. However, drawbacks are the relative high costs (especially when an MS-analysis is performed) and the inability of this method to serve for high throughput screening. Another limiting point becomes obvious when one compares the results of the two studies by Ho *et al* (2002) and Gavin *et al*. (2002): Their protein network maps differ significantly, probably because of their different tagging approaches, the choice of bait proteins, and last but not least possible variations in complex purification (von Mering *et al*., 2002).

3.2.3 Förster resonance energy transfer (FRET)

FRET occurs between two fluorescent molecules that are less than 10nm apart from each other. An excited donor chromophore submits energy to an acceptor chromophore by non-radiative transfer (Förster *et al*., 1948; Förster *et al*., 1951). A review of Piston *et al*. (2007) illustrates how this phenomenon can be adapted to study PPI and what advantages and limitations come with it. A common pair of fluorescent proteins used for tagging is CFP and YFP, despite their significantly overlapping spectra. This method has successfully been used to assay intracellular calcium (Miyawaki *et al*., 1997; Nagai *et al*., 2004), or cAMP activity (Ponsioen *et al*., 2004; Nagai *et al*., 2000). However, this methods also has certain disadvantages (Vogel *et al*., 2006): If the ratio of donor : acceptor exceeds 1:10 (or 10:1), the FRET signal cannot be observed due to the strong background of fluorescing molecules not undergoing FRET. Furthermore, the acceptor may be directly excited by the used light source, or the donor may leak into the detection unit. To avoid this problem, spectrally separated fluorophors can be chosen, however this usually leads to a decrease of the signal

intensity and therefore to a lesser detectability. Additionally, false negative results are quite common due to the localisation of the tags: In some cases two labelled proteins interact, however their fluorescent labels are on the opposite sides of the complex. Thus, the fluorophors are too far away from each other and no signal will be obtained.

3.2.4 Split ubiquitin

Split ubiquitin is a further enhancement of a two hybrid assay. Two halves of ubiquitin (Nub and Cub) are fused to the proteins of interest. Additionally, a LexA domain is fused to Cub. In case the two proteins of interest interact, Nub and Cub form a functional ubiquitin, resulting in the cleavage of the fusion protein and the release of LexA which in turn can start reporter gene transcription (Johnsson *et al*., 1994). The advantage over the common yeast two hybrid is that membrane proteins or other proteins excluded from the nucleus can be assayed. However, a drawback of this method is that a relatively high number of false positives is obtained.

3.2.5 Split GFP

Developed by Ozawa (2005), split green fluorescent protein (GFP) uses the phenomenon of protein splicing to re-unite two halves of GFP to a functional GFP protein. The N-terminal part of GFP and the N-terminal part of an intein, a protein that is able to excise itself and rejoin the remaining amino acids, is fused to protein of interest. The putative interaction partner is tagged with the C-terminal portion of the intein and the C-terminal part of GFP. Upon interaction of the proteins of interest, the close proximity of the two parts of the intein restores its function, leading to splicing of the two GFP parts, rejoining them and thus creating a fully functional GFP protein. The advantage of this method is that no additional reporter gene translation is needed, and PPIs can be monitored at any given place within a cell. However, a limitation of this method is that the splicing process takes about an hour, therefore transient events cannot be investigated (Ozawa 2005). Of course false negatives because of possible steric effects, and/or misfolding of recombinant proteins also have to be considered.

3.2.6 Fluorescence cross-correlations spectroscopy (FCCS)

FCCS is a method to study PPIs using different diffusion coefficient of the monitored complex components (Elson *et al*., 1974; Bacia *et al*., 2006). Two proteins of interest are labelled with two distinct fluorescent proteins which are then excited at a certain area within a cell. The fluorescence intensity fluctuation is then detected and measured by an optical unit. Those fluctuations are dependent on the diffusion coefficient of the protein or the protein complex. If the two proteins of interest are bound in one complex, their fluorescence fluctuation correlates, if the proteins are independent of each other, the signal will not. However, FCCS is not yet a well-established method in laboratories because of the following reasons: To analyse data acquired by FCCS, complex data processing by special software is required. Additionally, instruments, although commercially available are not yet standardized to allow the comparison of different experimental setups. To date, this method is also restricted by its inability to include membrane bound and therefore relatively static proteins, which play an important role in signal transduction. Furthermore, the two single proteins of interest have to differ in their diffusion coefficient to make the method work reliably.

4 Aim of the thesis

Despite immense effort in revealing the modality of MAPK signal transduction in budding yeast, some of the open questions have been proven difficult to be addressed. Particularly the dynamic interplay of factors of the SHO1 branch that leads to activation of signal transduction, feedback and signal specificity has not been well understood. The currently available methods to study PPI did not allow the investigation of membrane proteins. Moreover, the activation of this cascade seems to involve a number of transient PPI-events that could not be followed until now. Therefore, we made use of a recently developed enzymatic tagging approach to overcome some of these problems. This tagging assay is based on the transfer of a methyl group from a protein tagged with the catalytic domain of the murine histone methyl transferase (HMT) to a target protein that is tagged with a histone 3 acceptor domain. Using this assay we focussed on the following questions: First, we wanted to analyse how the composition of the SHO1 signalling complex changes upon pathway activation. Furthermore, we thoroughly investigated how the interaction of Ste11 with Sho1 is regulated and whether these events could possibly lead to signalling specificity. Last, we also investigate the dynamics of the recruitment of negative regulators to components of the HOG pathway.

5 Material and Methods

5.1 Primers

Rga1NheI: AGA GCT AGC CGT CAG GAT ATC TTT GTA
SalIFus1: AGA GTC GAC CCA GAA CCG CTA CTG AA
Fus1XbaI: GAG ATC TAG AGT CGT ATT CTT GGA GA
XhoISte20: GAG ACT CGA GTT GTG TTA CAT GCG AT
Ste20mitterev: AAG GAT TTT TCG AAG GAA AGC TTT
Ste20mittefw: ACC GTC CAA GCC TGA AGC AA
SalIRga1 GAG AGT CGA CTA ATC CAT ATT TGG T
Rga1mitterev: AGC GAA GGA TCC ATT TGG GA
Rga1mittefw: CGA AGG GTT CAA ATA CGG ATA TT
Ste20SpeI: GAG AAC TAG TCT TTT GTT TAT CAT CTT
PstIcdc42prom: GAG ACT GCA GGT TGC ATT ATT TCT ATC
cdc42promXbaI: GAT CTA GAC ATT TTG TGG AAG AGC TAA
XbaIcdc42: GAT CTA GAC AAA CGC TAA AGT GTG T
XbaIL4P: GAT CTA GAC AAA CGC CAA AGT CTC T
cdc42BamHI: GAG GAT CCC TAC AAA ATT GCA CAT T
XbaISuv: AGT CTA GAA TGG GAT CCT GTG TGC GTA T
SuvXbaI: CTC TCT AGA GAA GAG GTA TTT GCG G
PstIFus1orf: AGA CTG CAG ATG GTA GCA ACA ATA A
SalINpb2: AGA GTC GAC GGT CTT GTA GCC CTT TC AAT
Nbp2XbaI: GAT CTA GAA TCC GAT ATA TCT AAT TTT GTT
SalIOpy2: CTG TCG ACT TGT GCC CGA ACC AGG TGA TCA
Opy2XbaI: CTT CTA GAT CGT TCA TCG TGT ATT TCG AAC
Sho1-disrpt-C: CAG TGA AGC GGT TGC CTT TTT CTT TGT GAA CAT CCT AGA GCC TCG AGG CCA GAA GAC

Sho1kovorne: AAA GGG CGT GTT ATT AAC AGT AGC CAA CCC AAT AAT TTG AAA ACA ACT CCG GTT CTG CTG CTA

koMsb2vorne: TTC TGT TCA TCT CTA GCT GGC TTC CAC CTC GTT TCC TAT TTC CGG TTC TGC TGC TAG

koMsb2hinten: TAA GTT TAT AAG GTT ATG CAA GCG GAG AAA GTC TCT GCA GCC TCG AGG CCA GAA GAC

Hkr1kovorne: CCA CAA CTG ACG CAG TCA ACA GTC AGA AAC AGA GAA TGT ATC CGG TTC TGC TGC TAG

Hkr1kohinten: CCC CTT CCA GCA GCG TTT ATT GCA CAG TCA ATA ATC ATT TCC TCG AGG CCA GAA GAC

5.2 Plasmids

Plasmids used in this study can be found in table 1. Suv320 refers to HMTa and Suv324 refers to HMTi. All fusion proteins constructed in this study were made as follows: Genes of interest were obtained by PCR, introducing a SalI (SphI, XhoI) site upstream of the gene, and an XbaI (SpeI, NheI) site at their C-terminus. Cdc42 was tagged N-terminally by introducing the H3 or HMT tag via an XbaI site respectively.

plasmid	description	source
PC42	*ADH1* FRB-Suv320 YCp111	this study
PC43	*ADH1* FRB-Suv324 YCp111	this study
PC205	*ADH1* Fus1 pRS313	this study
PC159	*ADH1* Fus1-FKBP YCp22	this study
PC160	*ADH1* Fus1-Suv320 YCp111	this study
PC192	*ADH1* H3HA-FKBP YCp22	this study
PC157	Bem3-Suv320 YCp111	this study
PC181	Cdc42-H3HA YEp195	this study
PC165	Cdc42-Suv320 YCp111	this study
PC8	Hog1-Suv320 YCp111	I. Dohnal
PC233	Nbp2-H3HA YEp195	this study
PC446	Nbp2P/A-H3HA YEp195	this study
PC235	Nbp2-Suv320 YCp111	this study
PC303	Nbp2-Suv320 YEp181	this study
PC319	Opy2-H3HA 2µ	this study
PC267	Opy2-Suv320 YCp111	this study
PC1	Pbs2-FKBP YCp22	this study
PC7	Pbs2-Suv320 YCp111	I. Dohnal

PC280	Pbs2-Suv320 YEp181	this study
PC341	Pbs2-Suv324 YEp181	this study
PC448/GA1815	pFus1-LacZ	Gustav Ammerer
PC455	pStl1-LacZ	S. Salah
PC30	Rga1-Suv320 YCp111	this study
PC361	Rtn2-H3HA YCp33	S. Salah
E159	Sho1 YEp195	I. Dohnal
PC35/E227	Sho1-3xH3HA YEp195	I. Dohnal
PC38	Sho1D16H-H3HA YEp195	I. Dohnal
E225	Sho1-H3HA YCp33	I. Dohnal
PC36/E228	Sho1-H3HA YEp195	I. Dohnal
PC54	Sho1-Suv320 YCp111	this study
PC308	Sho1-Suv320 YEp181	this study
PC352	Sho1-Suv324 YEp181	this study
PC37	Sho1Y8A-H3HA YEp195	I. Dohnal
E348	Sho1Δ153-180-H3HA YEp195	I. Dohnal
E349	Sho1Δ181-213-H3HA YEp195	I. Dohnal
E350	Sho1Δ214-242-H3HA YEp195	I. Dohnal
E351	Sho1Δ243-268-H3HA YEp195	I. Dohnal
E532	Sho1Δ269-299-H3HA YEp195	I. Dohnal
PC40	Sho1ΔSH3-H3HA YEp195	I. Dohnal
PC358/Ap56	Ste11-H3HA Yep195	A. Zuzuarregui
PC359/Ap84	Ste11-Suv320 YCp111	A. Zuzuarregui
PC343	Ste20 Suv324 YEp181	this study
PC14	Ste20-Suv320 YCp111	this study
PC297	Ste50D/F-Suv320 YEp181	this study
PC172	Ste50-H3HA YEp195	this study
PC295	Ste50P/L-Suv320 YEp181	this study
PC18	Ste50-Suv320 YCp111	I. Dohnal
PC169	Ste50-Suv320 YEp181	this study
PC344	Ste50-Suv324 YEp181	this study

Table 1: Plasmids used in this thesis

The Rga1 gene and promoter were obtained by two PCRs using primer SalI/Rga1 and Rga1mitterev, and Rga1mittefw and Rga1NheI respectively.
The Ste20 gene and promoter were obtained by two PCRs using the primers ShoISte20 and Ste20mitterev, and Ste20mittefw and Ste20SpeI respectively.
The Cdc42 promoter was obtained by PCR using primers PstICdc42prom and Cdc42promXbaI; the Cdc42 gene was made using the primers XbaICdc42 and Cdc42BamHI.
The L4P mutation was obtained by using the primer XbaIL4P.
The ORF of Fus1 was obtained by PCR with the primers PstIFus1orf and Fus1XbaI.
Suv320 without stop codon was obtained by PCR using the primers SuvXbaI and XbaISuv.
Suv320 and 324 for N-terminal tagging of Cdc42 were obtained using the primers XbaISuv and SuvXbaI.
Fus1 promoter and gene were obtained by using primers SalIFus1 and Fus1XbaI.
Opy2 promoter and gene were obtained by using primers SalIOpy2 and Opy2XbaI.
Fus1 P/A gene was obtained by using Fus1 primers on p4263 of C. Boone's plasmid collection. The W/S and P/A W/S double mutation were obtained the same way, using p4597 and p4598 as templates respectively.
Nbp2 was created using the primers SalINpb, and Nbp2XbaI. Nbp2 P/A as obtained by two PCRs: the first part by using primers SalINbp2 and Nbp2 P/A to front; the second part by using primers Nbp2P/Anachhinten and Nbp2XbaI.
The Ste50 D/F and P/L mutation was introduced by cutting pKT655 (H. Saito) Sfu/BglII and inserting this mutation containing piece in the plasmid PC239 which contains a wild-type Ste50 gene. From there this mutation was used for further processing.

pbs2FKBP (pC1): pC1 was constructed by removing the GFP of pVR15 (Vlado Reiser) by XbaI and inserting FKBP from the vector PC4EN-F1 (ARIAD) via XbaI/SpeI.
All other FKBP fusion proteins were constructed by exchanging pbs2 in the vector Pbs2 FKBP YCp22 (I. Dohnal) with the respective gene plus promoter via SalI (or SphI)/XbaI, cutting the insert with those or compatible restriction enzymes.
All HMT fusion proteins were constructed by exchanging Pbs2 in the vector Pbs2 Suv YCp111 or YEp181 (I. Dohnal) with the respective gene plus promoter via SalI (or SphI)/XbaI, cutting the insert with those or compatible restriction enzymes.
All H3 fusion proteins were constructed by inserting the respective gene in the vector H3HA YEp195 (PC150) via SalI (or SphI)/XbaI, cutting the insert with those or compatible restriction enzymes.

Since **Cdc42** needs N-terminal tagging, the cloning procedure for this gene was different: For all fusion constructs: first the vectors YCp111 and YCp22 were cut PstI/BamHI and the promoter (PstI/XbaI) and gene (XbaI/BamHI) of Cdc42, both in a pGEM vector, were inserted by triple ligation. FKBP and Suv were inserted into the XbaI site: FKBP by XbaI/SpeI from PC4EN-F1 and Suv by XbaI from a pGEM plasmid. For the H3 fusion, first YEp195 was cut PstI/BamHI and Cdc42 promoter and gene were inserted, then the H3 tag was inserted from Ap35 by XbaI.

***ADH1* FRB SUV (**pC42/43) was constructed by first inserting an *ADH1* promoter from the plasmid K2381 SphI/EcoRI, and the FRB gene from the plasmid pC4-RHE via EcoRI/SpeI. Then *ADH1* FRB YCp111 was used as vector and cut SpeI to insert Suv320 or 324, cut out of pCF7 (320) pCF3 (324) by XbaI/SpeI.

For the construction of ***ADH1* H3HA YCp22 pC192** Pbs2 was exchanged for the *ADH1* promoter plus H3 tag from Ap35 by cutting vector XbaI/SphI and insert SpeI/SphI.

The ***ADH1*** promoter and the **Fus1** was first combined in the YCp22 vector by triple ligation of the empty vector (HindIII/XbaI), the *ADH1* promoter (HindIII/PstI) and the Fus1 gene in a pGEM vector (PstI/XbaI). Fusion proteins were then made as follows: For the Suv-fusion, the vector Pbs2Suv320YCP111 was cut EcoRI/XbaI, the *ADH1* Fus1 was inserted by HindIII/XbaI and the Suv was inserted again by HindIII/EcoRI. The FKBP fusion was obtained by swapping Pbs2 in the Pbs2FKBPYCp22 vector for *ADH1* Fus1 by XbaI/HindIII, and the H3 fusion was made by inserting Fus1 also via XbaI/HindIII in H3HA YEp195.

5.3 Strains

All strains used in this study are derivates of W303 (Rothstein *et al*., 1983) and can be found in table 2.

Strain	Genotype	Source
W303	*Mat a, ade2, trp1, can1, leu2, his3, ura3*	K. Nasmyth
Rapa^R	*Mat a tor1-1 fpr1::NatMx*	S. Gruber
YC7	*Mat a ste50::KanMx*	P. Dahl
YC163	*Mat a, opy2::KanMx*	this study
YC183	*Mat a, msb2::his*	this study
YC186	*Mat a, hkr1::KanMx*	this study

YC189	*Mat a, hrk1::KanMx, msb2::his*	this study
YC210	*Mat a, ste20::trp*	this study
YC241	*Mat a, nbp2::trp*	this study
YC247	*Mat a, nbp2::trp, pbs2::his*	this study
YC76	*Mat a. pbs2::his*	I. Dohnal
YC93	*Mat a, ste11::ade*	A. Zuzuarregui
YC100	*Mat a, sho1::trp*	this study
YC247	*Mat a, nbp2::trp, ssk1::his*	this study
YC197	*Mat a, ssk1::his*	this study
SS89	*Mat a, hog1::KanMx*	S. Salah

Table 2: Strains used in this thesis

The knock-out of Sho1 with trp was obtained by homologous recombination of a cassette obtained by using the primers sho1kovorne and sho1-disrpt-C using GA2264 as a template.
YC163 (*Δopy2*) was constructed using the primers SalIOpy2 and Opy2XbaI on the Opy2::KanMx from the Euroscarf collection and using this PCR product for homologous recombination in W303
YC175 (RapaR sho1::KanMx) was obtained by homologous recombination by a PCR product obtained by the primers sho1kovorne and sho1-disrpt-C using GA2264 as a template.
YC183 (msb2::his): This k.o. of msb2 was obtained by recombination with a PCR product; primers used are msb2ko vorne and msb2ko hinten, the template was GA2260.
YC186 (hkr1::KanMx) is a k.o. of hkr1 by recombination with a PCR product obtained by the primers hkr1ko vorne and hkr1ko hinten using the template GA2259.
YC189 (msb2::his hkr1::kan) is a k.o. of hkr1 in msb2::his by recombination with a PCR product obtained by using the primers hkr1ko vorne and hkr1ko hinten; the template was GA2259
YC210 (Ste20::trp) was obtained by using the primers ste20ko vorne und hinten and template the GA 2264 to create a PCR product that was used for homologous recombination
YC241 (nbp2::trp) is a k.o. of nbp2 created by homologous recombination using a PCR product obtained by using the template GA 2264 and the primers nbp2 k.o. front and nbp2 k.o. back.
yC247 (nbp2::trp pbs2::his) was constructed by homologous recombination with PCR product obtained by the primers nbp2 k.o. front and nbp2 k.o. back and using the template GA2264.

5.4 Methods

Yeast transformation for homologous recombination:

An over night culture of the strain to be transformed is diluted to OD_{600} of 0.2 and grown to 0.6 to 1. Cells are centrifuged at 2000 rpm for 3minutes in 50 ml tubes, and washed with H_2O. They are resuspended in LiAc/TE (10µl 1M LiAc, 10µl 10x TE, 80 µl H_2O), transferred in a 1,5 ml tube and mixed with 620µl of PEG buffer (60µl LiAc, 60µl 10xTE, 480 µl 50% PEG 3350, 10µl salmon sperm) and 10µl of the knock out cassette-DNA. After vortexing the sample is incubated at 30° C for 30 minutes and 70 µl DMSO are added, followed by an incubation of 15 minutes at 42° C. The sample is placed on ice for one minute, and then centrifuged at 4000 rpm for 3 minutes. In case of auxotrophy genes, the cells are directly plated on selective media, whereas for KanMX knock-outs, regeneration in YPD medium is done for 2 hours prior to plating.

One-step yeast transformation:

To introduce plasmids into yeast cells the following protocol was used:
Cells and plasmid DNA are added to 150µl of one-step buffer (0.2M LiAc, 40% PEG4000, 100mM DTT) and mixed by vortexing. The sample is incubated at 45°C for 45 minutes, and then plated on the respective selective media.

Salt stress treatment:

An over night culture is diluted to OD_{600} of 0,05 - 0.2 and grown to 0.4 to 1. The sample is split, and pre-warmed NaCl is added to one half to a final concentration of 0.4M, the other half is supplemented with the same volume of pre-warmed H_2O. Both are kept shaking at 30°C for 45 minutes, and are then harvested at 2500rpm for 2 minutes.

Protein isolation:
Depending of the protein read out, two different methods of protein isolation were used.

NaOH-protein-extract:
The cell pellet is resuspended in 200 µl 1 % Glucose and transferred in a 1,5 ml tube. 200 µl 0,5 M NaOH is added, and the sample is incubated at room temperature for 3 minutes. After

centrifugation at 13.000 rpm, the supernatant is discarded, and 1x sample buffer (50mMTris pH7,5, 2 % SDS, 10 % Glycerol, 100mM DTT) is added. The samples are boiled at 95° C for 3 minutes and after another centrifugation step and the transfer of the protein containing supernatant, the protein concentration is determined by measuring OD 260/280.

Proteins isolation via beads:
1-2 µl Protein breaking buffer (50mM Tris pH 8, 50mM NaCl, 0,5 % Tween 20, 0,1 % SDS) is added to the cell pellet per mg of pellet, and the same amount of glass beads is added. The samples are broken up by 'Fastprep' for two times 20 seconds, intensity 6.0. Samples are then centrifuged at full speed for 15 minutes, and the supernatant is transferred into a new 1,5 ml tube. An aliquot is taken to determine protein concentration by measuring OD 260/280, the rest of the sample is mixed with 2x urea sample buffer (40mM Tris pH6,8, 8M Urea, 5 % SDS, 0,1mM EDTA, 1 % MeEtOH) and boiled at 95° C for 3 minutes.

Immunoprecipitation (IP):

50 ml liquid cultures were grown to an OD_{600} of ~0,8 and harvested by centrifugation for 2 minutes at 2500 rpm. 500 µl breaking buffer (50mM HEPES pH8, 140mM NaCl, 1mM EDTA, 1 % Triton, 10mM PMSF, complete protease inhibitor mix 1 tablet/50 ml) and 200µl glass beads are added and cells are broken by Fastprep for two times 45 seconds at intensity 4,5. After centrifugation at 4° C and 13000 rpm for 15 minutes the supernatant is transferred into a clean Eppendorf tube and protein concentration is estimated.
30 µl of Dynabeads Pan Mouse IgG (Invitrogen) were used per IP-sample. Beads were rinsed with 0,5 % BSA in PBS and incubated with 25 µl HA antibody in 500 µl PBS-BSA. Beads were rotated at 4° C for 8 hours. For crosslinking beads were washed three times with 0,2 M NaBorat pH 9 and afterwards incubated for 30 minutes at room temperature with 20mM DMP in 0,2 M NaBorat (pH 9). Beads were then washed three times with 250 mM Tris (pH8) and then equilibrated with protein isolation buffer.
IP was done over night at 4° C, and after three washing steps with protein isolation buffer 20 µl SDS protein loading buffer was added to the beads and boiled for 5 minutes prior to loading of the protein gel.

Western Analysis:

1.5 mm thick acrylamide gels are run at 200V and 30-50 mAmp per gel using the following running buffer: 14.4g glycine, 3g Tris, 1g SDS per litre. Two times three Whatman papers, as well as the nitrocellulose membrane and the gel are wetted with transfer buffer (25mM Tris pH8, 0.192M Glycerine, 0.02% SDS), and the blotting sandwich is built, having 3 Whatman papers at the bottom followed by the membrane, the gel, and 3 more Whatman papers on top. Depending on the size of the protein to be detected, the time of blotting varied between 1,5 and 3 hours, and the current between 50 and 90 mAmp per gel. After the transfer, the membrane is stained with Ponceau S to verify the transfer. The colour is washed off by rinsing the membrane with PBS-T (for one litre: 8g NaCl, 0,2 g KCl, 1,45 g Na_2HPO_4, 0,24 g KH_2PO_4, ph7,4) and blocked 3 hours to overnight in 5% skim milk powder in PBS-T. After blocking, the membrane is rinsed with PBS-T and incubated with the antibodies. For H3K9 detection, the membrane is incubated 2 hours to over night with the primary and secondary antibody together, together with 100µl/ml wild type yeast protein extract (mg pellet = µl PBS-T = µl glass beads; 2x 45 sec fastprep, intensity 4,5; after centrifugation at full speed for 15 minutes take the supernatant). For HA detection primary and secondary antibody is diluted in 0,5 % skim milk in PBS-T and incubated for one hour. After washing the membrane for at least 3x 10 minutes in PBS-T, the signals are detected by ECL (34 mg p-Coumaric acid, 226 mg Luminol, 100ml Tris pH 8 per litre)

Quantitative β-Gal Assay:

Cells were grown until the growth rate was exponential and then subjected to salt treatment. When harvested by centrifugation at 2500 rpm, OD_{600} of cells is measured. The cell pellet is resuspended in 665 µl H-Buffer (10mM HEPES pH7, 150mM NaCl, 2mM $MgCl_2$, 1 % BSA) and 55µl of each chloroform and 0,1 % SDS are added. The samples are vortexed for 1 minute, and incubated shaking at 37° C after the addition of 125 µl ONPG solution (4mg/ml ONPG in H-buffer). As soon as the sample turns yellow, the reaction is stopped with 400 µl Na_3CO_3. Samples are then centrifuged at 13000 rpm for 5 minutes, and OD_{420} is then measured. Miller units are then calculated:

Miller units = $(1000*OD_{420})/(t*v*OD_{600})$

Where t is the time of the colorimetric reaction and v is the volume of culture harvested.

5.5 Sequences

Sequence of the HMT SET domain used for the methylation assay: grey marks the differences of the active and inactive HMT.

```
tctagaatgggatcctgtgtgcgtatcctcaagcagttccacaaggacttagaaagggag
S  R  M  G  S  C  V  R  I  L  K  Q  F  H  K  D  L  E  R  E
ctgctccggcggcaccaccggtcaaagacccccggcacctggacccaagcttggccaac
L  L  R  R  H  H  R  S  K  T  P  R  H  L  D  P  S  L  A  N
tacctggtgcagaaggccaagcagaggcgggcgctccgtcgctgggagcaggagctcaat
Y  L  V  Q  K  A  K  Q  R  R  A  L  R  R  W  E  Q  E  L  N
gccaagcgcagccatctgggacgcatcactgtagagaatgaggtggacctggacggccct
A  K  R  S  H  L  G  R  I  T  V  E  N  E  V  D  L  D  G  P
ccgcgggccttcgtgtacatcaatgagtaccgtgttggtgagggcatcaccctcaaccag
P  R  A  F  V  Y  I  N  E  Y  R  V  G  E  G  I  T  L  N  Q
gtggctgtgggctgcgagtgccaggactgtctgtgggcacccactggaggctgctgcccg
V  A  V  G  C  E  C  Q  D  C  L  W  A  P  T  G  G  C  C  P
ggggcgtcactgcacaagtttgcctacaatgaccagggccaggtgcggcttcgagccggg
G  A  S  L  H  K  F  A  Y  N  D  Q  G  Q  V  R  L  R  A  G
ctgcccatctacgagtgcaactcccgctgccgctgcggctatgactgcccaaatcgtgtg
L  P  I  Y  E  C  N  S  R  C  R  C  G  Y  D  C  P  N  R  V
gtacagaagggtatccgatatgacctctgcatcttccgcacggatgatgggcgtggctgg
V  Q  K  G  I  R  Y  D  L  C  I  F  R  T  D  D  G  R  G  W
ggcgtccgcaccctggagaagattcgcaagaacagcttcgtcatggagtacgtgggagag
G  V  R  T  L  E  K  I  R  K  N  S  F  V  M  E  Y  V  G  E
atcattacctcagaggaggcagagcggcggggccagatctacgaccgtcagggcgccacc
I  I  T  S  E  E  A  E  R  R  G  Q  I  Y  D  R  Q  G  A  T
tacctctttgacctggactacgtggaggacgtgtacaccgtggatgccgcctactatggc
Y  L  F  D  L  D  Y  V  E  D  V  Y  T  V  D  A  A  Y  Y  G
aacatctcccgctttgtcaaccacagttgtgaccccaacctgcaggtgtacaacgtcttc
inactive: actttgtcaacaag
N  I  S  RH F  V  N  HL S  C  D  P  N  L  Q  V  Y  N  V  F
atagacaaccttgacgagcggctgccccgcatcgctttctttgccacaagaaccatccgg
I  D  N  L  D  E  R  L  P  R  I  A  F  F  A  T  R  T  I  R
gcaggcgaggagctcacctttgattacaacatgcaagtggaccccgtggacatggagagc
A  G  E  E  L  T  F  D  Y  N  M  Q  V  D  P  V  D  M  E  S
acccgcatggactccaactttggcctggctgggctccctggctcccctaagaagcgggtc
T  R  M  D  S  N  F  G  L  A  G  L  P  G  S  P  K  K  R  V
cgtattgaatgcaagtgtgggactgagtcctgccgcaaatacctcttctctaga
R  I  E  C  K  C  G  T  E  S  C  R  K  Y  L  F  S  R
```

Sequence of the 4xH3 3xHA tag

One of the four H3-repeats is highlighted in grey.

```
gggatcccgaattcggctcgtactaagcagaccgctcgcaagtccaccggcggcaaggcc
 G   I   P   N   S   A   R   T   K   Q   T   A   R   K   S   T   G   G   K   A
ccgcgcaagcagctggcccagatcccgaattcggctcgtactaagcagaccgctcgcaag
 P   R   K   Q   L   A   Q   I   P   N   S   A   R   T   K   Q   T   A   R   K
tccaccggcggcaaggccccgcgcaagcagctggcccagatcccgaattcggctcgtact
 S   T   G   G   K   A   P   R   K   Q   L   A   Q   I   P   N   S   A   R   T
aagcagaccgctcgcaagtccaccggcggcaaggccccgcgcaagcagctggcccagatc
 K   Q   T   A   R   K   S   T   G   G   K   A   P   R   K   Q   L   A   Q   I
ccgaattcggctcgtactaagcagaccgctcgcaagtccaccggcggcaaggccccgcgc
 P   N   S   A   R   T   K   Q   T   A   R   K   S   T   G   G   K   A   P   R
aagcagctggcccagatctgcggccgcatcttttacccatacgatgttcctgactatgcg
 K   Q   L   A   Q   I   C   G   R   I   F   Y   P   Y   D   V   P   D   Y   A
ggctatccctatgacgtcccggactatgcaggatcctatccatatgacgttccagattac
 G   Y   P   Y   D   V   P   D   Y   A   G   S   Y   P   Y   D   V   P   D   Y
gctgctcagtgcggccgc
 A   A   Q   C   G   R
```

6 Results

6.1 The methylation assay – general parameters and the application of the assay to the Sho1-Pbs2 PPI

Since dynamic recruitment of signalling factors to the plasma membrane is of particular importance for HOG pathway activation, we needed an assay that allows tracking of such events. However, as described in the introduction (chapter 3.2.), currently available methods to study PPI do not allow to measure transient PPIs of membrane proteins. To address this problem, our lab has developed a novel PPI assay based on enzymatic tagging (see below). Initial PPI experiments were performed by I. Dohnal (see below and thesis I. Dohnal) and showed first promising results. In the work presented here, the assay was further adapted and improved and applied to study the dynamic events upon activation of the SHO1 branch.

6.1.1 Characterisation of the methylation event

Enzymatic tagging is defined as the transfer of a chemical modification that can easily be detected with an appropriate readout system. This transfer is dependent on an acceptor and a donor and can only be catalysed when the two partners are in close vicinity. Our tagging approach is based on the murine histone methyl transferase (HMT) Suv39h1 serving as donor, and its substrate histone 3 lysine 9 (H3K9) acting as acceptor (Rea *et al*., 2000). Both proteins were fused to the putative interaction partners (the proteins of interest), respectively. An important feature of the HMT is that it should theoretically not interfere with the yeast host system, because there is no endogenous mechanism for methylation of H3K9 in *S. cerevisiae*. However, it should be mentioned that the tail of the yeast histone H3 is highly homologous to the histone tail of mammalians and therefore could possibly also serve as an acceptor for methylation by the murine HMT. Nevertheless, we strongly believe that our system does not influence the host organism because of the following reasons: For our tagging approach we made use of the amino acids 82-412 of the HMT, containing only the catalytic domain that is sufficient for the methylation of the substrate K9, however, this version of the HMT does not contain any binding motifs (Rea *et al*., 2000) and should therefore not be able to localise to the histones of the host cell.

For our enzymatic tagging assay an additional mutation (H320R) at the catalytic centre of the HMT was introduced, resulting in a hyperactive allele (HMTa). This mutant allele accepts free histones as substrate in *in vitro* assays, showing that the catalytic activity of the HMTa is independent of its localisation and of co-factors (Rea *et al*., 2000). As a negative control, an additional mutant allele, H324R, was chosen, in which the enzymatic activity is completely abolished (Rea *et al*., 2000) (HMTi). As a substrate (acceptor) a 4x repeat of amino acids 1-21 of the murine histone H3 (containing the substrate K9) was fused to one of the proteins of interest. This acceptor domain was found to be sufficient in preliminary experiments (I. Dohnal, PhD thesis). Additionally, to easily assess the expression of the substrate protein, a 3x HA repeat was added.

We used Western blotting as a readout system, because compared to other methods it is an inexpensive, time-extensive method that does not require special equipment. Furthermore, Western blotting is not only a direct readout, but also potentially allows quantification of measured PPIs. Different types of antibodies for methylated H3K9 were available. In the beginning of our studies we used a polyclonal rabbit anti-multi-methyl-H3 antibody, kindly supplied by the lab of T. Jenuwein (antibody 2236, in this thesis referred to as α-m(TJ)). However, unfortunately this antibody lost its specificity during the course of this project. Therefore, a new monoclonal antibody against methylated H3K9 was developed (generated and kindly provided by the lab of E. Ogris), which was used for later results of our studies (designated α-m(EO) in this study).

In general, proteins subjected to our studies were either tagged with HMT or H3 at their C-terminus (with the exception of Cdc42 which was tagged at the N-terminus) and expressed from plasmids under the control of their endogenous promoters in a W303 strain background. However, depending on each individual experiment, different knock out strains were used, and sometimes proteins had to be expressed from either centromeric or 2μ plasmids.

With this method, we established a direct readout assay and we were able to study transient PPIs at the plasma membrane in the HOG pathway. Initial cloning of HMT and H3 tag and preliminary experiments to verify that PPIs can be detected using this assay were carried out by I. Dohnal and are described elsewhere (PhD thesis Ilse Dohnal). These experiments focussed on Sho1 and Pbs2, which were previously described to interact upon osmostress.

6.1.1.1 Kinetics and temperature sensitivity of the methylation event

We first wanted to investigate, whether our assay is sensitive to temperature shifts, because enzymatic reactions are influenced by temperature changes. Furthermore, the HMT used for these studies is derived from mouse and the *in vitro* methylation assays performed by Rea *et al.* (2000) were performed at 37° C, whereas we wanted to make use of the HMT in yeast, an organism that has its optimal growth temperature at 30° C. For these assays we added a conditional component to our methylation assay: We made use of the two proteins FKBP and FRB that interact constitutively upon addition of rapamycin (Choi *et al*., 1996). The HMT was fused to FKBP and the Histone tag to FRB, both fusion constructs were expressed under control of the alcohol dehydrogenase promoter (*ADH1*) from centromeric plasmids (Fig. 6A). Using this system, cells should only accumulate a methylation signal upon the addition of rapamycin. This is because only when the HMT and the H3 tag are tethered together via the induced FRB-FKBP interaction, the proteins should be close enough for the HMT to methylate the H3 substrate.

First, we subjected our negative control (HMTi) to this treatment. As seen in figure 6B, no methylation signal was accumulated when the inactive methylase mutant was used. Next, we wanted to test if the system responds differently on increasing temperatures when the HMTa allele was used: Interestingly, slight signals could already be detected without the addition of rapamycin compared to the negative control. This indicates that the two proteins FRB and FKBP have enough affinity towards each other to interact slightly even without rapamycin induction. Alternatively, one could assume that the methylase and the H3 tag retain a certain affinity. However, the accumulated signal may also be due to the high abundance of the fusion proteins, since they were both expressed by the *ADH1* promoter. When cells were grown on 4° C, no methylation signal could be accumulated upon the addition of rapamycin. However, methylation rates increased with increasing temperature. Whereas at 23° the saturation of methylation signal needed at least 120 minutes, we already observed saturation after 40 minutes when cells were grown on 37°.

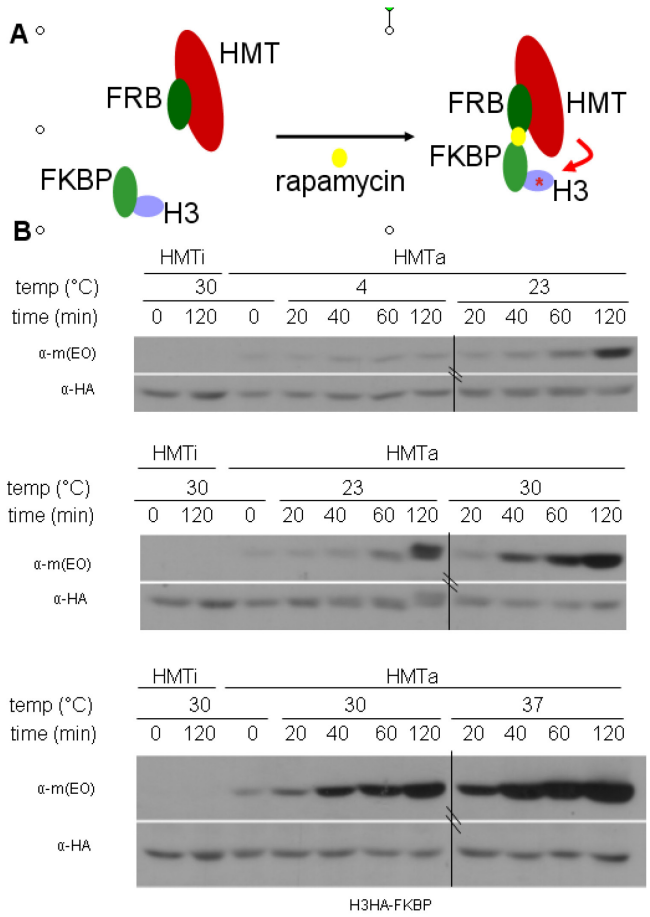


Figure 6: Temperature sensitivity of the methylation assay: **A** Cartoon of the conditional assay: The HMT is fused with FRB and H3HA is fused with FKBP. Upon addition of rapamycin FRB and FKBP interact, resulting in the methylation of the H3 tag by the HMT. **B** *ADH1*-H3HA-FKBP CEN and *ADH1*-FRB-HMT CEN were co-expressed in a Rapa^R strain and subjected to rapamycin treatment at different temperatures.

These results indicate that the murine histone methyl transferase works just fine in yeast; however, it is also evident that temperature conditions have to be constant or should be taken into account.

6.1.1.2 The methylation assay is not sensitive to oxidative stress nor to salt stress up to a concentration of 0,6 M NaCl

We wanted to investigate whether the methylation system is a suitable approach to study PPIs of the HOG pathway. Therefore, we needed to rule out that the assay is sensitive to NaCl in the experimental concentrations. To test this, we again used the rapamycin induced conditional system. Rapamycin treatment was combined with the addition of NaCl in increasing concentrations, and signal accumulation was monitored for one hour. Western analyses of the experiment using 0, 0,2 and 0,4 M NaCl were quantified, but no significant differences in accumulation of methylation signals were observed (Fig.7A, B). Further experiments revealed that this accumulation was independent of NaCl up to a concentration of 0,6 M. Only at higher salt concentrations accumulation rates decreased significantly (Fig. 7C). We concluded that the HMT is insensitive to NaCl up to 0,6 M. However, concentrations beyond 0,8 M NaCl inhibit methylation signal accumulation significantly.

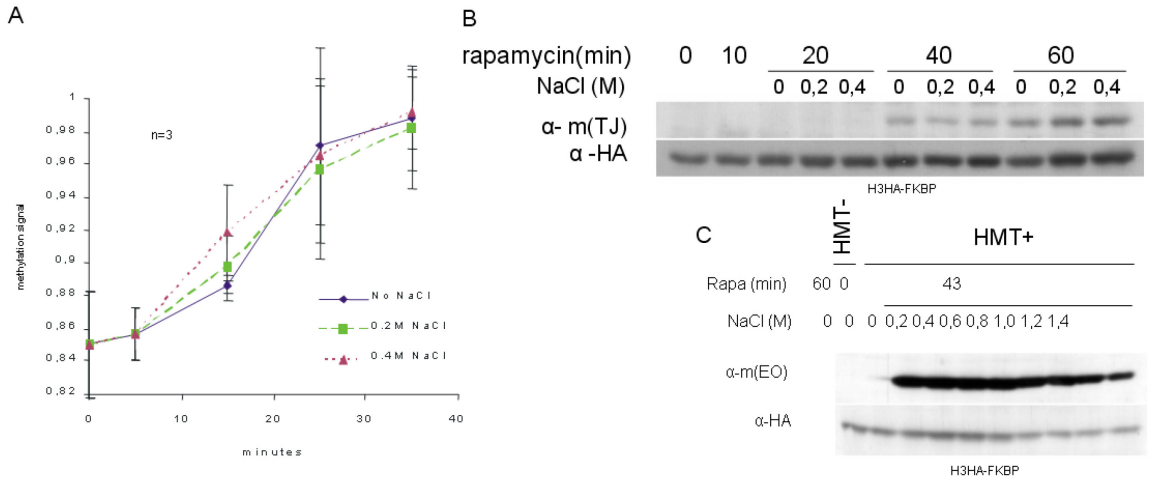


Figure 7: The methylation assay is not sensitive to NaCl. Both, FKBP-H3HA and HMT-FRB are expressed from a CEN plasmid and under the control of the *ADH1* promoter. Experiments were performed in a Rapa^R strain. **A** Quantification of the Western blot analysis. **B** A representative Western analysis that was used for quantification. After treatment with rapamycin for ten minutes, cells were split and subjected to salt treatment in different concentrations. **C** Methylation signal after 40 minutes at 0 – 1,4 M NaCl.

The methylation system was also subjected to oxidative stress, using H_2O_2 as the agent of choice. Addition of H_2O_2 did not lead to any accumulation of methylation signal when added simultaneously with rapamycin. However, when rapamycin was added 10 minutes prior to the addition of H_2O_2, signal accumulation occurred at similar rates as in cells treated only with rapamycin (Fig. 8). This indicates that H_2O_2 does not influence the methylation *per se*, but rather blocks the rapamycin induced interaction of FRB and FKBP. Thus, we believe that the methylation assay is also a suitable means for studying PPIs during oxidative stress. However, if the rapamycin inducible component is used, rapamycin has to be added at least 10 minutes prior to the addition of H_2O_2.

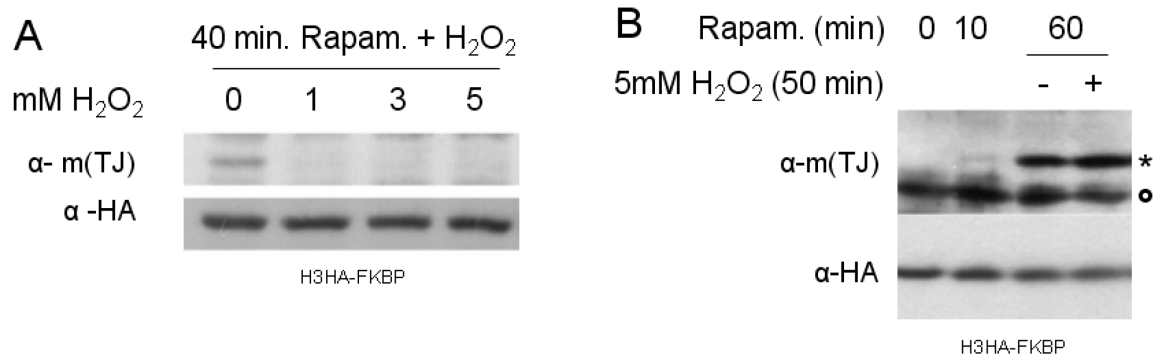


Figure 8: Analysis of the effect of H_2O_2 on the methylation assay. A rapamycin was added at the same time as H_2O_2, in **B** rapamycin was added 10 minutes before treatment with H_2O_2. * indicates the specific band, ° indicates an unspecific band.

6.1.2 Testing the methylation assay on the Pbs2-Sho1 interaction

6.1.2.1 The methylation signal correlates with the affinity of Sho1 and Pbs2 in a series of affinity mutants

Pbs2 and Sho1 are both essential proteins for osmostress signalling via the SHO1 branch of the HOG pathway (see chapter 3.1.4.5.). We first tested the methylation assay using this pair of proteins, because previous experiments have clearly shown that they interact with each other. Interaction of the two proteins was shown to be crucial for the activation of Pbs2 by Ste11 (Tatebayashi *et al*., 2003). Furthermore, different mutants of Sho1 that have an altered affinity towards Pbs2 have been available. Therefore, we could additionally assess if the methylation signal correlates with the affinity of the protein pair. The different alleles used in this study were Y8A, D16H and Y54M (Marles *et al*., 2004) and a ΔSH3 mutant. Those mutants were published to have decreased (Y8A, D16H,) or completely inhibited (ΔSH3) association with Pbs2 (It should be mentioned that in the original paper of Marles *et al*. there seems to be a discrepancy: Although the K_D of the D16H mutation (6,3 µM) is smaller than the K_D of the Y8A mutant (13,8µM), the decrease of the activating phosphorylation of Hog1 has been found to be stronger reduced in the D16H mutant). H3 fusion proteins of the Sho1 wild-type, the affinity mutants and the ΔSH3 mutant allele were assayed for the ability to interact with Pbs2-HMT.

This experiment was performed in both, wild-type and *Δsho1* strains in parallel. As seen in figure 9A we were able to obtain methylation signals for the interaction of Sho1 and Pbs2. For wild-type Sho1 we obtained similar signals in the wild-type and in a *Δsho1* strain. However, the signal was completely abolished when using the ΔSH3 allele in the *Δsho1* strain background. Interestingly, it was still present in the wild-type strain, although significantly weaker than when the wild-type Sho1 allele was used. These results can be explained by the fact that Sho1 aggregates as homo-oligomers at the plasma membrane (Hao *et al*., 2007): In wild-type yeast the endogenously expressed wild type Sho1 is present and heterogeneous complexes might be formed by wild-type and mutant Sho1. In the case of the affinity mutant, endogenous Sho1 helps to recruit Pbs2 to the membrane bound aggregates, resulting in methylation signals in wild-type yeast compared to *Δsho1* strains, where only the mutant allele is expressed. This experiment strikingly shows that close vicinity of proteins is sufficient to generate a methylation signal, which is a drawback of this method to study PPI. However, it is a common problem to all established assays to

investigate PPIs that close vicinity and not direct interaction is sufficient to generate a readout signal.

Next, we asked the question, if the interaction of Sho1 and Pbs2 changes upon exposure to osmostress. As seen in figure 9B (experiment performed by I. Dohnal) methylation signals were readily obtained with Sho1 wild-type and mutants. However, no significant change of methylation signal comparing stressed and non-stressed protein samples was observed. This result was rather unexpected, because the interaction of Sho1 and Pbs2 is known to be essential during osmostress. Therefore, we wondered, if maybe the method itself needed to be improved to allow finding of potential differences concerning the interaction of Sho1 and Pbs2. Two different strategies to improve the assay are reported in the following two chapters: On one hand, we tried different commercially available antibodies; on the other hand we introduced the previously described conditional component to our system.

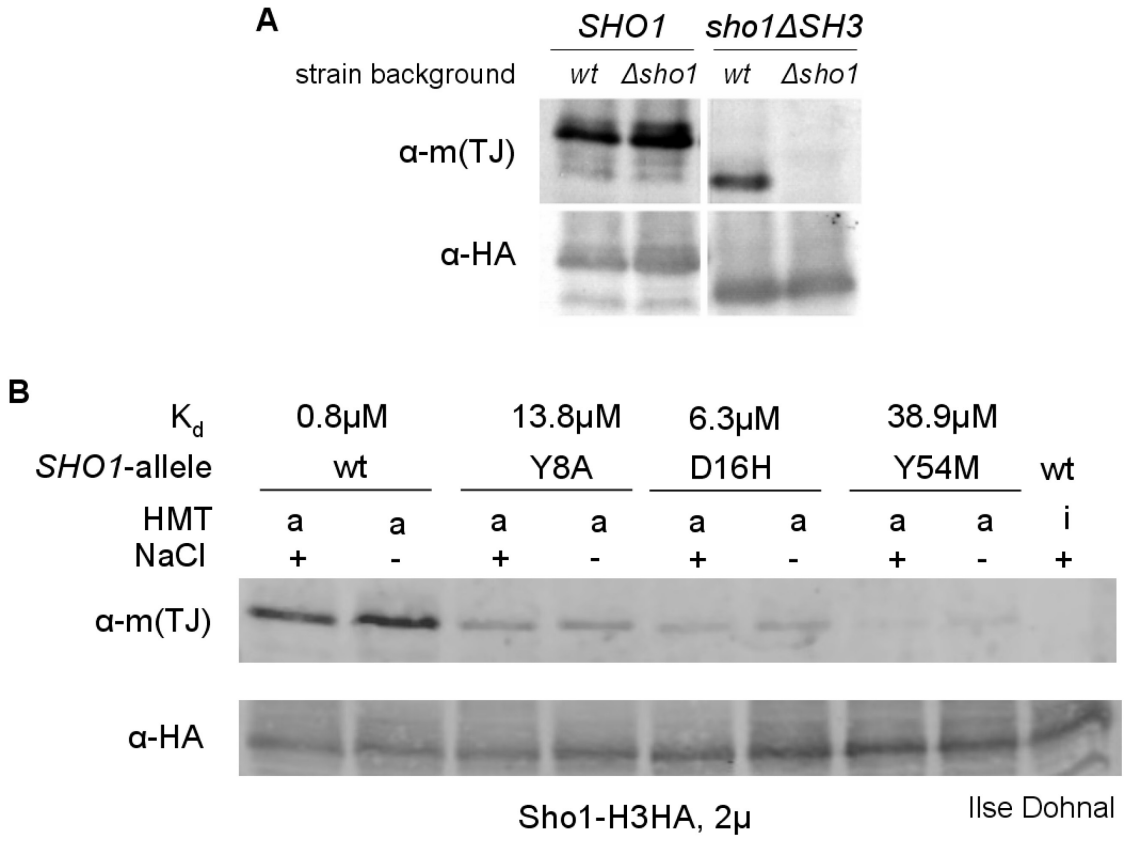


Figure 9: The interaction of Pbs2/Sho1 of wild-type Sho1 and affinity mutants. A The interaction of Pbs2-HMT CEN and Sho1-H3 2µ wild-type and affinity mutants in wild-type or *Δsho1*. **B** Protein Coexpression of Pbs2-HMT CEN and wild-type as well as mutant Sho1-H3 2µ. Salt stress was performed for five minutes (I. Dohnal, PhD thesis).

6.1.2.2 Trimethylation of the H3 tag is specific for protein interaction

Lysine 9 of the murine Histone H3 can be methylated at three different positions of the nitrogen atom. Depending on how many methyl groups become attached the modification can be defined as mono-, di- or tri-methylation. We reasoned that these three different enzymatic reactions of mono- di- and tri-methylation may all have different kinetics, making them more or less suitable for our experiments. Three antibodies that specifically recognize the different types of methylations are commercially available (Abcam). We reasoned that one of the different commercially available antibodies could be more suitable to track transient interaction of the Sho1-Pbs2 PPI. The previously used antibody mainly recognizes trimethylation of the H3 tag, and we figured that the kinetics of the trimethylation step could be too slow for transient interactions. We therefore reasoned that maybe mono- or dimethylation steps might be faster and may still be able to be catalysed even during those short-lived interactions. Additionally to the HMT and the H3 tag, we introduced the FRB-FKBP inducible components to the assay (Fig. 10A). For this system, three fusion proteins were needed: Sho1 was tagged with H3, Pbs2 was fused to FKBP. The third plasmid expressed an FRB-HMT fusion protein under the control of the *ADH1* promoter. Using this system, no methylation signal should accumulate without the addition of rapamycin, because even if Sho1 and Pbs2 interact, the HMT will not be bound to the same protein complex and therefore will not be able to modify the H3 tag. Only upon rapamycin treatment the HMT will become tethered to Pbs2 via the FRB-FKBP interaction, leading to subsequent methylation of the H3K9.

To test the different available antibodies we used untagged Sho1 (Fig. 10B lane 1) and Pbs2-HMTi (lane 2) as negative controls, and the above described rapamycin inducible system before (lane 3) and after (lane 4) induction. As expected, the different antibodies showed different patterns of methylation. None of the antibodies reacted on cell extracts not containing the H3 tag (lane 1) or containing the HMTi (lane 2) suggesting that they do not recognize unmethylated H3. Furthermore, all three commercially available antibodies gave signals even before the addition of rapamycin when the inducible system was used (lane 3). The mono-methyl antibody did not show any increase of the signal upon rapamycin treatment (lane 3 and 4), indicating that this modification is rapid and can already be saturated by random interactions, possibly caused by the high abundance of the HMT, since it was constitutively expressed by the *ADH1* promoter. The di-methyl antibody also showed a strong signal even without inducing the system. However, it did increase upon addition of rapamycin. α-m(TJ), an antibody mainly recognizing trimethylation, showed the largest

increase of the methylation signal. The commercially available tri-methylation antibody also showed a slight increase. However, since the differences in signal intensities were lower than the ones observed with α-m(TJ), it is likely that the commercial antibody isn't specific for trimethylation, but also recognizes mono- or di-methylation.

These results convinced us to continue our experiments with the α-m(TJ) (later α-m(EO)) antibody, because it was the antibody that showed us the strongest increase in methylation signal upon rapamycin treatment. Furthermore, the antibodies against mono- and di-methylation gave signals before induction with rapamycin and were therefore not suitable to study the Sho1-Pbs2 interaction.

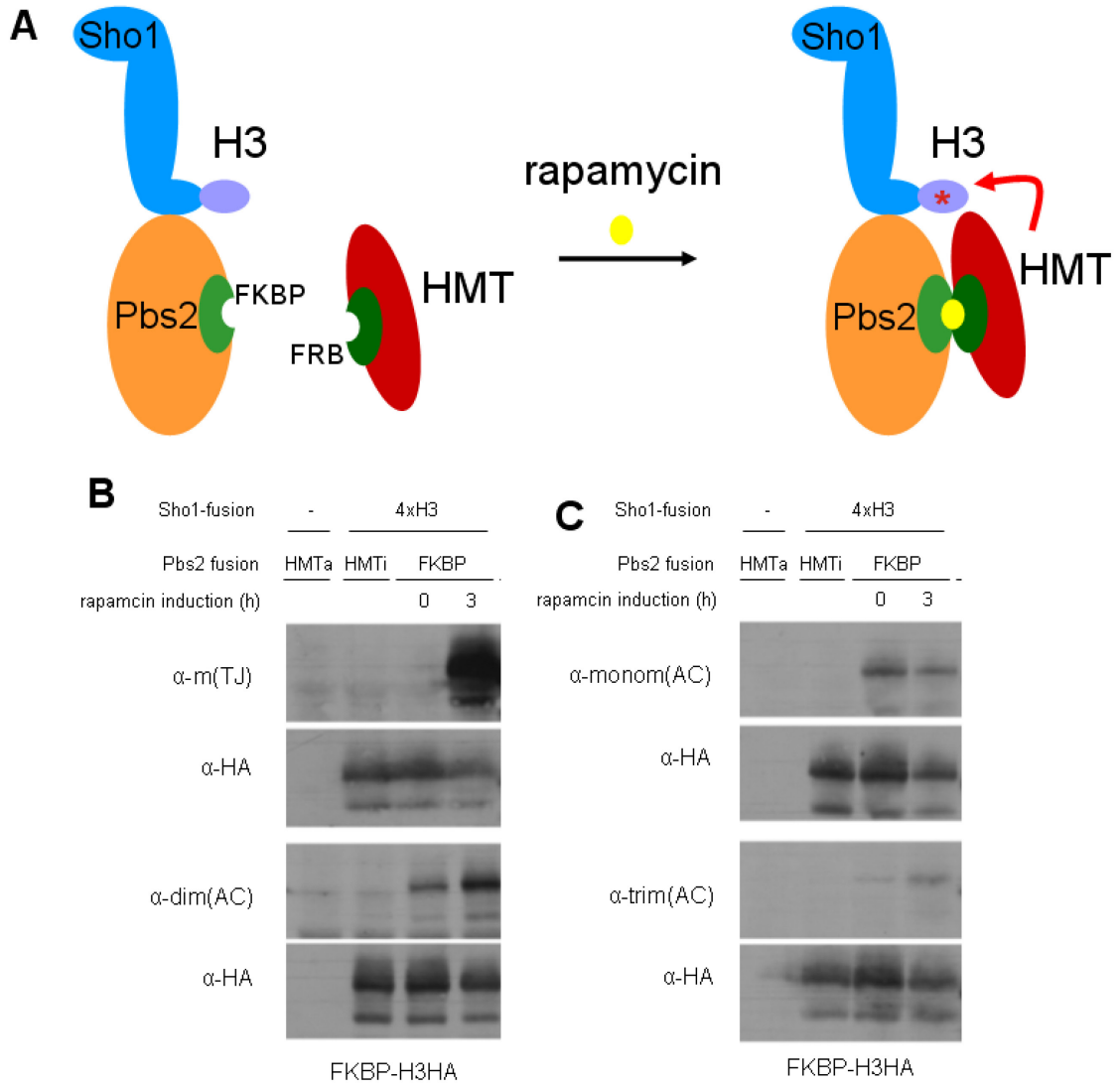


Figure 10: **Comparison of different available antibodies by western analysis of the Sho1/Pbs2 interaction. A** A scheme of the conditional system, see text for information. **B, C** Western analysis comparing different antibodies. The same protein extracts were used on the four western blots. Sho1-H3 fusions were expressed from a 2μ-plasmid, Pbs2-HMT or -FKBP fusions from a CEN plasmid. Lane 1: untagged Sho1 and Pbs2-HMT, lane 2. Sho1-H3 and Pbs2 tagged with HMTi; lane 3 and 4: Sho1-H3 and Pbs2-FKBP without or after 3 hours of rapamycin treatment.

6.1.2.3 Osmostress decreases the interaction rate between Pbs2 and Sho1

Next, we wanted to see if the inducible system (described in chapter 6.1.1.) will allow us to track transient PPIs. We figured that the steady-state methylation signal of the Sho1-Pbs2 interaction accumulated under non-stress conditions masks transient changes caused by osmostress. Therefore, the inducible system will help to avoid the accumulation of methylation signal before the start of the experiment (e.g. in our case application of osmostress). To prove that not only the methylation signal intensities, but also the methylation accumulation rates correlate with the affinity of the binding partners, the affinity mutants Y8A and D16H (described in chapter 6.1.2.) were also analysed using the conditional rapamycin induced system. As seen in figure 11A, accumulation of methylation signal could readily be detected upon addition of rapamycin. However, the accumulation rate was lower in the affinity mutants compared to wild-type. This result showed that the methylation signal as well as the methylation rate correlates with the affinity of the studied proteins.

To study differences in the Sho1-Pbs2 interaction upon pathway activation, we used the rapamycin inducible conditional system in combination with salt stress: Cultures were induced with rapamycin for 90 minutes to allow the accumulation of a signal above noise. Subsequently, the samples were split: One half was subjected with NaCl, the other half with H_2O. As shown in figure 11B, salt stress led to a delayed accumulation of methylation signal compared to unstressed samples, possibly representing lower interaction rates of Sho1-Pbs2 when the HOG pathway was activated. This suggested that either the interaction of Sho1 and Pbs2 was reduced upon osmostress or that the interaction dynamics changed in a way that methylation accumulation was reduced.

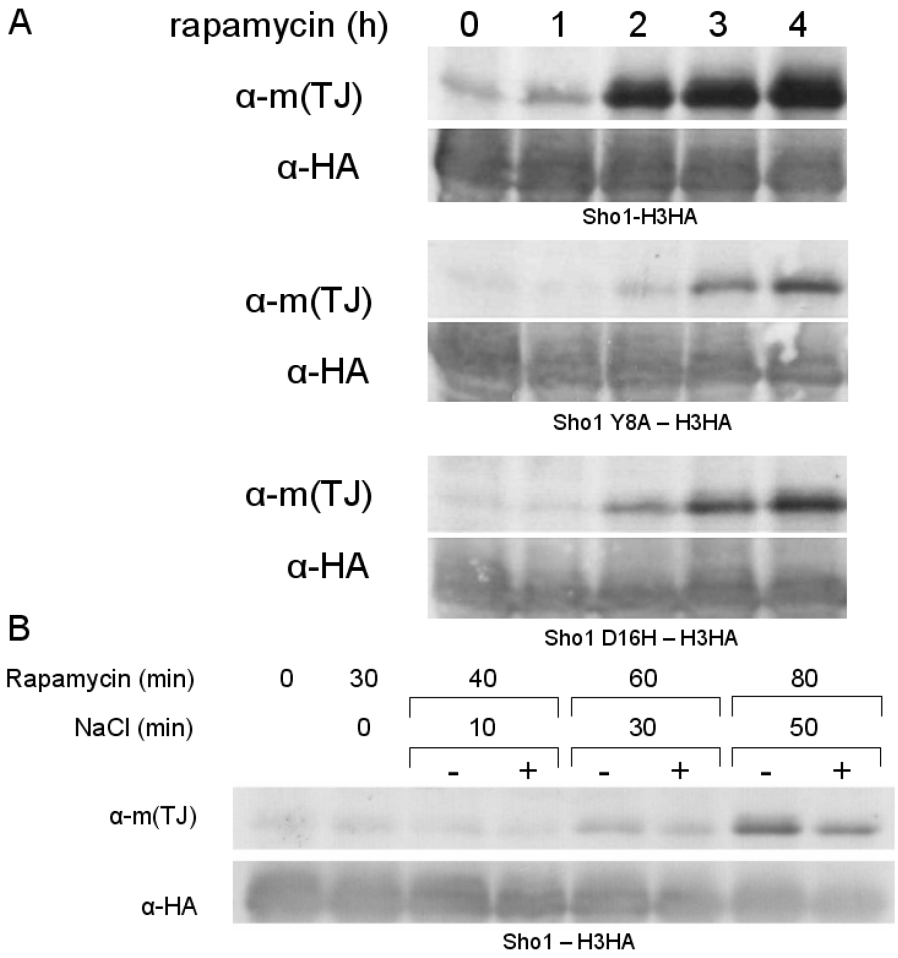


Figure 11: Sho1/Pbs2 interaction upon salt treatment. The inducible methylation system applied to Sho1-Pbs2 interaction in a Rapa^{R} strain: Coexpression of Sho1-H3, Pbs2-FKBP and *ADH1*-FRB-HMT. **A** Comparison of Sho1 wild-type and mutants using the rapamycin inducible system: Cultures were subjected to rapamycin treatment, and methylation accumulation was monitored. **B** Application of osmostress: The sample is treated with rapamycin and split after 90 minutes. Half of the culture was treated with 0,4 M NaCl, the other half was treated with water.

6.2 Protein-protein interactions at the plasma membrane

As described in the introduction, HOG signalling is activated by two individual input branches (see introduction chapter 3.1.4.5.). While the two-component system of the SLN1 branch is well understood, the complexity of the SHO1 branch has made a detailed understanding of the branch architecture and signalling dynamics difficult. A number of proteins have been identified that play an important role to generate proper SHO1 branch signalling. However, the spatial and temporal interplay of the components before and after activation of the branch has been until now not clearly understood. We tried to address this problem by studying the possibility of interactions of several components of the SHO1 branch, focussing on the membrane associated proteins. Several initial experiments analysing the interactions of components of the SHO1 branch bound to the plasma membrane were performed and will be summarized in the following chapter.

6.2.1 Interaction of Sho1 and Cdc42 with different components of the HOG pathway

Sho1 has been described to be an essential protein in an SLN1 signalling defective strain background. It may have two roles in the signalling cascade: It is part of the osmosensing machinery, but also seem to have important function as an adaptor protein. Cdc42 is an essential G-protein, playing a role in three MAPK pathways in yeast (see chapter 3.1.4.5.). Cdc42 is also anchored at the plasma membrane and as a crucial activator of the signalling cascade it is likely to tether downstream components, possibly by forming the core of a signalling complex. We were interested if and when these proteins become associated with the other signalling components of the SHO1 branch and whether these interactions contribute to signalling specificity. We believed that both, Cdc42 and Sho1, might possibly anchor different signalling complexes to the plasma membrane during signal transduction.
Seven factors that have been associated with SHO1 signalling were tagged with HMT and assayed for interaction with Sho1-H3 (Fig 12A). These factors were the MAPK Hog1, the MAPKK Pbs2, the PAK-like kinase Ste20, the Ste11-adaptor protein Ste50, the Cdc42 GTPase activating proteins (GAPs) Rga1 and Bem3, and the membrane protein Fus1 (see chapter 3.1.4.5.).

We found a strong interaction of Sho1 with Pbs2 (which served as a positive control) and Fus1. Interaction of Sho1 with Ste20 or Rga1 showed clearly visible but less intensive signals. Interestingly, Bem3 and Ste50 produced no interaction signals with Sho1.

These observations made us conclude the following: First, it indicated that the different affinities of the GAPs (Rga1 and Bem3) may play a role in pathway specificity, since both regulate the activity of Cdc42, but only Rga1 becomes recruited to the complex formed by Sho1. Furthermore, it supported the idea that Ste50-Sho1 interaction possibly is a very transient event and therefore not detectable with the methylation assay. However, when both constructs were overexpressed, a distinct signal was observed (Fig. 14B). Additionally, the MAPK Hog1 itself was found to interact with Sho1 even non stress conditions showing that most tested proteins involved in salt stress signal transduction have a certain affinity to Sho1 independent of stress application.

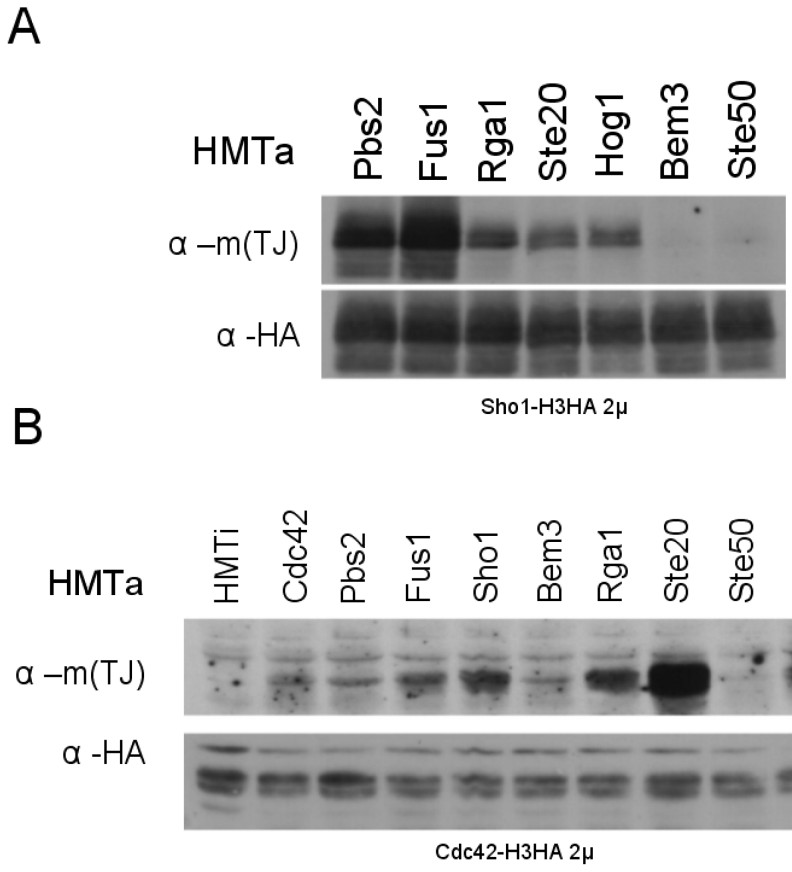


Figure 12: Interaction of Sho1 and Cdc42 with different components of the Hog pathway. A Western Analysis of Sho1-H3 2µ with different HMT fusions in CEN plasmids. **B** Western Analysis of Cdc42-H3 2µ and different HMT CEN fusions. Experiments were performed in a wild-type strain.

To investigate which proteins of the HOG pathway can interact with Cdc42 before stress application, we performed similar interaction experiments with Cdc42-H3 under normal growth conditions: As expected, Ste20 strongly interacted with Cdc42 independently of salt stress (Fig. 12B). Cdc42 has been previously described to be required for recruiting Ste20 to sites of polarised cell growth (Leberer *et al*., 1997). Moderate interaction signals have been found with Sho1, Rga1 and Hog1. Signals slightly above background were found with Pbs2, Bem3 and Cdc42 itself. However, as it was the case for

the Sho1-Ste50 interaction, methylation signals could be detected, when both, Cdc42 and Ste50 fusion proteins were expressed from a 2µ plasmid (Fig 13D).

This result shows that Cdc42 is already associated with HOG specific components like Pbs2 and Hog1 under normal growth conditions. Additionally, the interaction signal of Cdc42 with itself suggests that it possibly forms multimers. This experiment further supports the idea that different GAPs of Cdc42 may contribute to signalling specificity: While only Rga1, but not Bem3 showed interaction with Sho1, both, Rga1 and Bem3, show a methylation signal with Cdc42. This indicates that although both GAPs associate with Cdc42, only Rga1 associates with the Sho1 signalling complex.

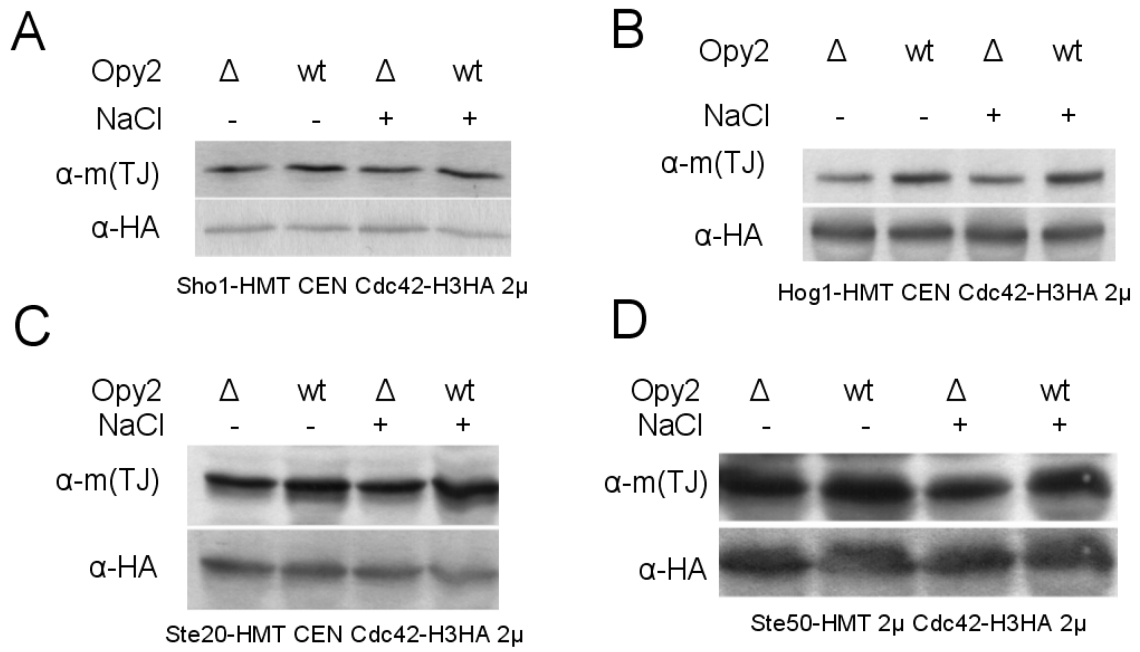


Figure 13: The impact of salt stress and Opy2 on Cdc42-H3 2µ interaction capabilities. Cdc42-H3 is expressed from a 2µ plasmid in either wild-type or *Δopy2*. **A** Coexpression with Sho1-HMT CEN. **B** Coexpression with Hog1-HMT CEN, **C** Coexpression with Ste20-HMT CEN **D** Coexpression with Ste50-HMT 2µ.

We next wanted to analyse, how induction of the signalling cascade by osmostress changes the composition of the membrane bound complex. Therefore, we assayed the interaction of Cdc42 with its binding partners. We were wondering whether we would be able to observe an increase of the interaction signals of proteins of the SHO1 branch (Sho1, Ste20, Ste50) with Cdc42 upon pathway activation. Such an increase would be an indication that a regulation of the affinity of Cdc42 to components of the SHO1 branch upon osmostress contributes to signalling specificity. However, the interaction of Cdc42 with these proteins did not significantly alter upon application of osmostress (Fig. 13, compare wild-types). Furthermore, the interaction signals of Cdc42 and Hog1 also showed

no difference. Hog1 has been described to translocate into the nucleus upon activation. Therefore, Hog1 should no longer be associated with the membrane-bound complex. However, since we did not use the rapamycin inducible component for this experiment, a potential decrease in affinity could not be detected.

These results indicated that Cdc42 has no increased affinity towards the tested binding partners upon pathway activation by osmotic stress. Therefore the mechanism that guarantees specificity of the osmostress signal mediated by the activity of Cdc42 remains elusive.

6.2.2 Targeting Ste50 to the plasma membrane

The phosphorylation of Pbs2 by Ste11 is the step crucial to determine signalling specificity in the HOG pathway, because while Ste11 acts as MAPKKK in three different MAPK cascades, Pbs2 is specific for osmostress response. However, while the upstream components of this signalling cascade are plasma membrane associated, Ste11 is thought to be localised in the cytoplasm (Huh *et al*., 2003). Therefore, the targeting of Ste11 to the membrane bound complex is a crucial step in osmostress signal transduction. This targeting process is thought to mainly depend on the adaptor protein Ste50. Therefore, we asked the question, if Ste50 interacts with different membrane associated proteins, and whether the interaction rate changes upon osmostress. The three different tested membrane proteins were Cdc42 (which is employed in three MAPK pathways), Sho1 (a potential osmosensor that also acts in the FG pathway), and Opy2 (an adaptor protein that is essential for the SHO1 branch) (see chapter 3.1.4.5.). We tested whether we would be able to find interactions of these three proteins with wild-type and two mutant alleles of Ste50: The D146F (D/F) substitution is located in a conserved, central domain, and causes Ste50 to become constitutively recruited to the plasma membrane, possibly due to constitutive interaction with Sho1 (Tatebayashi *et al*. 2006). This mutant allele of Ste50 is able to activate the HOG pathway in combination with a catalytically constitutively active Ste11 allele without stress application. The P318L (P/L) mutation is located in the RA domain of Ste50 and abolishes Ste50 binding to Cdc42 and is therefore incapable of signalling.

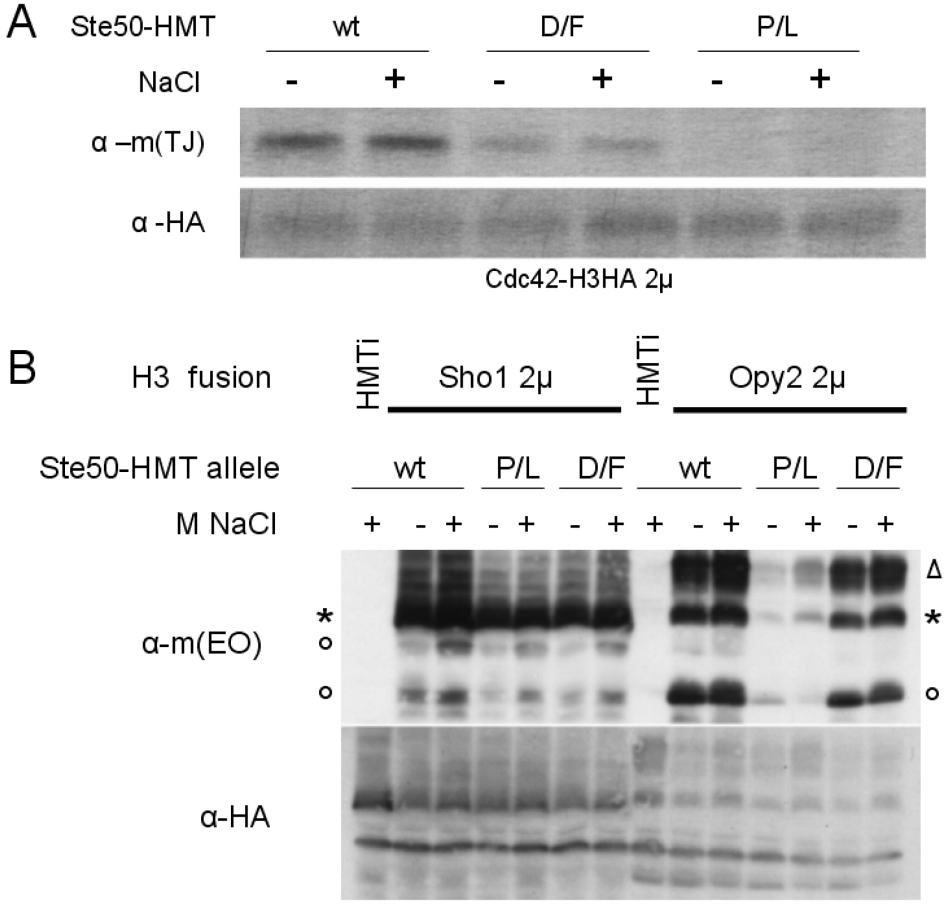


Figure 14: Ste50 and its interaction partners. A Interaction of Cdc42-H3 2μ with Ste50-HMT 2μ wild-type and mutants in *Δste50*. **B** Ste50-HMT 2μ interaction with Sho1-H3 2μ and Opy2-H3 2μ in *Δste50*. * indicate the main band, ° indicate specific degradation products, Δ indicate aggregates.

First, we assayed the interaction of Cdc42 with the different Ste50 alleles. In agreement with Tatebayashi *et al.* (2006), the P/L mutant showed a strongly reduced interaction to Cdc42 (Fig. 14A). Interestingly, the interaction signal of the D/F mutant with Cdc42 was also significantly lower compared to the signal obtained with wild-type Ste50. This was surprising to us, because we expected constitutive recruitment of this mutant to the plasma membrane and therefore an increase of the methylation signal of Ste50D/F to Cdc42. This observation can be an indication that either the D/F mutant negatively influences the binding to Cdc42, or that the increased affinity of the D/F mutant to Sho1 (or a yet unknown factor) indirectly lowers the affinity between Cdc42 and Ste50.

Next we analysed the interaction of Sho1 and Ste50. If Sho1 and Cdc42 indeed compete for Ste50 binding, then the P/L mutant, incapable of interacting with Cdc42, could give increased signals with Sho1 compared to wild-type. Furthermore, if the D/F mutant is truly constitutively recruited to Sho1, a stronger interaction signal of Sho1 and Ste50D/F compared to wild-type should be obtained as well. However, both mutants showed no

increase, but rather a slight decrease of the methylation signal. This result indicated neither that neither Sho1 competes with Cdc42 for Ste50 binding nor that the D/F mutant constitutively binds to Sho1.

To further analyse how the D/F mutant may possibly constitutively recruit Ste11 to the plasma membrane, we finally asked, if Opy2 is involved, for it was shown also play a role in the process of targeting Ste50 and therefore Ste11 to the plasma membrane (the function of Opy2 in SHO1 signalling and its possible interaction partners will be explained in the next chapter). Therefore, we assayed the interaction of Opy2 with the three different Ste50 alleles. Surprisingly, the P/L mutant showed strongly reduced interaction signals compared to wild-type. However, the D/F mutant showed no differences. This result indicates that the inability of Ste50 to bind Cdc42 also strongly affects its ability to bind Opy2. However, the mechanism of how the D/F mutant accomplishes to constitutively recruit Ste11 to the plasma membrane remains elusive.

6.2.3 Opy2 and its impact on the HOG pathway

As already in chapter 3.1.4.5., Opy2 was recently found to have an impact on HOG pathway signalling (Wu *et al*., 2006). Being a transmembrane protein, it may serve as a membrane anchor for cytoplasmic proteins. Indeed, using the methylation assay, a slight influence of Opy2 on the interaction of Cdc42 with other proteins could be detected. As shown in figure 13, Sho1, Ste20, Hog1 and Ste50 appear to have slightly lower methylation signals with Cdc42 in the *Δopy2* strain compared to wild-type. Thus, Opy2 might have an influence on the interaction of Cdc42 with the respective proteins, independent of exposure to salt stress.

To investigate how Opy2 may influence the system we studied the interaction signals between Opy2 and Ste11, Ste50, Sho1 (figure 15A). When tagging Opy2 with the HMT, no differences in signal intensities in response to salt stress could be observed with respect to all four the partner proteins tested. However, when tagging Opy2 with H3-HA, a slight increase of methylation signal was detected upon osmostress with all of them (Fig. 15B). Additionally to the major band representing full length Opy2, a second, lower band of a potential degradation product could be detected. Interestingly, this band showed methylation intensities contrary to the intact protein. Stronger signals were obtained without stress compared to the signals of the stressed protein samples. However, while protein levels of the full length protein showed roughly the same abundance during stress and non-stress conditions, the abundance of the degradation product is strongly decreased

during stress conditions. This may hint to a higher turnover of Opy2 during vegetative growth, and a stabilization of the protein upon application of salt stress.

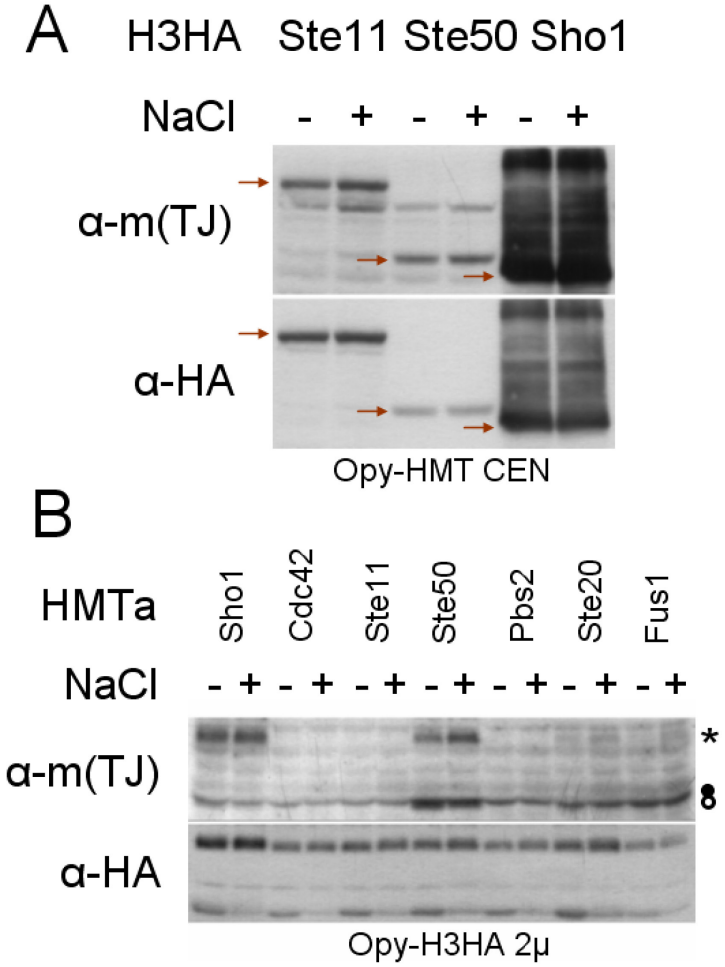


Figure 15: Interaction partners of Opy2. A Opy2-HMT CEN coexpressed with Ste11, Ste50 and Sho1 tagged with H3 2µ respectively. Arrows indicate the main bands. **B** Opy2-H3 2µ coexpressed with different proteins tagged with HMT CEN (exception Ste50-HMT 2µ). Experiments were performed in a wild-type strain. * indicates the main band, °indicates a specific degradation product, • indicates an unspecific band.

6.2.4 Activated Hog1 increases the abundance of Rtn2

Rtn2 is a member of the reticulon protein family. Reticulons are an evolutionary conserved protein family that are mainly associated with the endoplasmic reticulum (ER). They were shown to be of importance for bending and shaping the ER, and furthermore for trafficking, and vesicle formation (reviewed in Yang *et al*., 2007).

Interestingly, Rtn2 was found to be of importance for the SHO1 branch in a screen by V. Reiser and S. Salah (unpublished data): *Δrtn2 Δssk1* double deletion strains were found to be salt sensitive, indicating that Rtn2 is necessary for full activation of an osmoresponse via the SHO1 branch. Since Rtn2 has not yet been described in the context of osmostress signalling, we wanted to test, whether Rtn2 is a not yet identified component of

the SHO1 branch. Therefore, we were curious to see whether it interacts with any component of the Sho1 signalling complex.

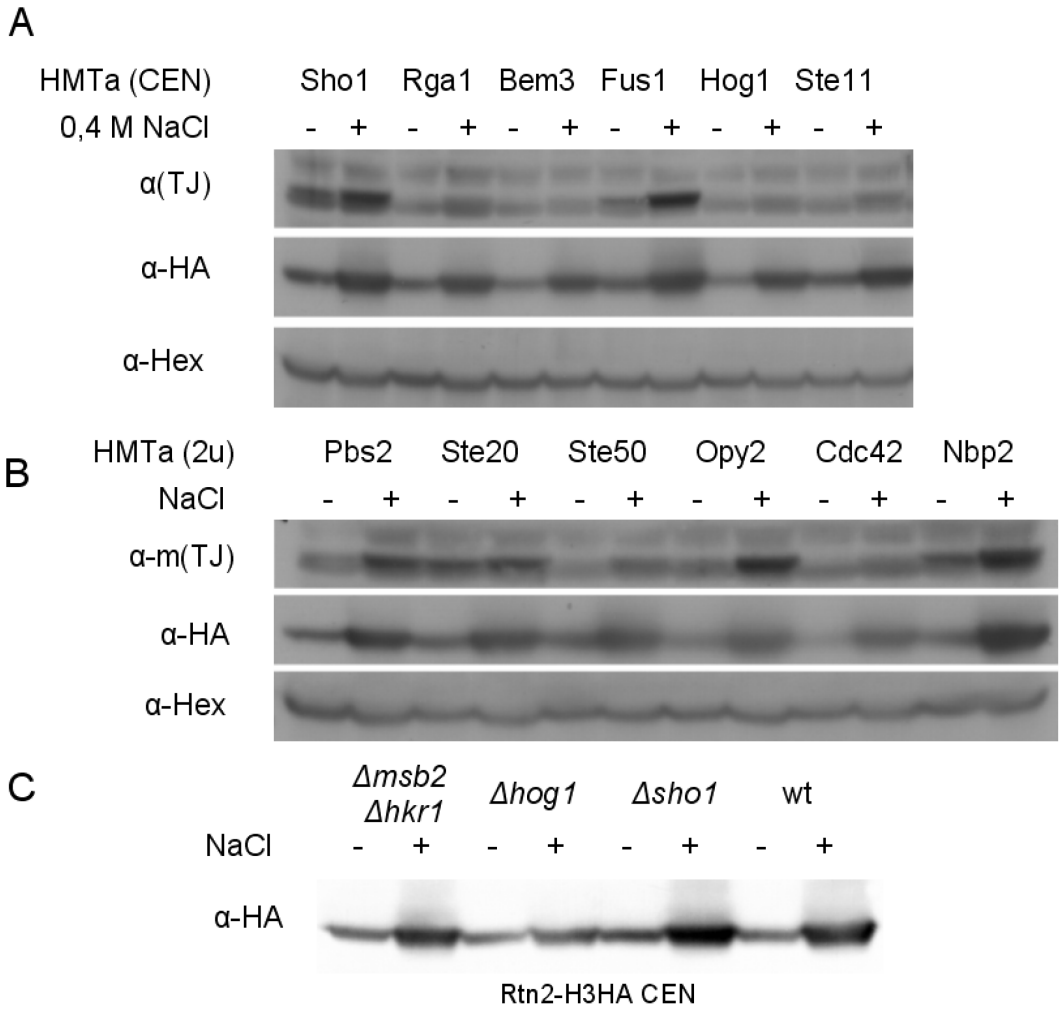


Figure 16: Rtn2-H3 CEN interacts with components of the HOG pathway. Co-expression of different HMT fusion proteins with Rtn2-H3 CEN. The HMT tagged proteins are expressed from a CEN plasmid (**A**) or from a 2µ plasmid (**B**). Experiments were performed in a wild-type strain. **C** Rtn2-H3-HA CEN expressed in different strains.

We analysed Rtn2-H3 with HMT-fusions of most of the known proteins involved in SHO1 signalling. Interestingly, we discovered that Rtn2 protein levels are influenced by osmostress: Upon addition of salt, Rtn2 protein levels rose significantly (Fig. 16). Furthermore, this increase in Rtn2 abundance was Hog1-dependent, since in a *Δhog1* deletion strain protein levels of Rtn2 did not change. This observation indicated that Hog1 is required to either stabilize the Rtn2 protein, and/or to enhance its transcription (Fig. 16C). Regarding the interaction to the factors of the SHO1 branch, we observed an interaction signal during normal growth conditions for some (if not all) tested fusion proteins. Upon osmostress application, an increase in the interaction signal was obtained for almost all tested fusion proteins. However, we believe that the elevated protein levels of

Rtn2 may cause this increase in the methylation signal. Nevertheless, there seems to be one exception: No increase of the Rtn2-Ste20 interaction upon osmostress was observed, despite the increased protein levels of Rtn2 after stress treatment. This suggests an actual decrease of interaction rate of Ste20 and Rtn2 upon salt stress.

In summary, these results show that upon osmostress treatment, protein levels of Rtn2 rise and lead to increased interaction signals with components of the SHO1 branch, probably indicating that the interaction rates of the protein pairs do not change. Since interaction signals of Ste20 and Rtn2 do not change upon stress treatment despite the risen protein levels of Rtn2, one may conclude that the interaction rate of these two proteins decreases upon pathway activation. However, more experiments will be required to show how Rtn2 contributes to the function of the Sho1 branch and whether the interaction of Rtn2 and Ste20 plays a crucial role in signal transduction.

6.3 The interaction of Ste11 and Sho1 in the HOG pathway

6.3.1 Ste11-Sho1 interaction increases upon osmostress

As already described in the introduction of this thesis the MAPKK Ste11 becomes activated during osmotic stress, and also during the response to pheromone and during starvation conditions (see chapter 3.1.4.6.). Therefore, it has been a field of intense research to identify mechanisms that guarantee signal specificity of the individual MAPK cascades in budding yeast. A current model suggests that some components of the HOG pathway sequester Ste11 upon osmotic shock and thereby channel the signal directly to the downstream MAPKK Pbs2. One of the candidate factors discussed is the membrane bound protein Sho1. Therefore, we wanted to analyse whether Ste11 and Sho1 can interact. We also wanted to identify factors that can possibly influence this interaction.

Indeed, in our initial experiments Ste11 and Sho1 were found to interact under normal growth conditions (Fig. 17, lane 2). Furthermore, the methylation signal increased slightly in intensity upon salt stress (Fig. 17, lane 3). These observations indicated that Sho1 sequesters Ste11 upon osmostress and that this interaction could cause Ste11 to become deprived from other possible interaction partners, which otherwise could lead to inaccurate activation of the other MAPK pathways.

6.3.2 Ste11-Sho1 interaction under osmostress is increased in *Δpbs2* and *Δhog1* cells

Since Pbs2 is thought to have an important scaffolding function in the HOG pathway, we tested whether the Ste11-Sho1 interaction depends on the presence of Pbs2. As shown in figure 17A, this interaction is not abolished in *Δpbs2* cells. In contrast, the interaction signal is strongly enhanced under stress conditions in this strain background. Semi-quantification allows the estimation of a more than four-fold increase of the signals compared to wild type (Fig. 17B). These results indicate that the presence of Pbs2 is not required for the interaction of Sho1-Ste11. On the contrary, the affinity of the two proteins is higher in the absence of Pbs2. These results can be interpreted in two ways: The increase of the methylation signal in the *Δpbs2* mutant can either be caused by the lack of the scaffolding function of Pbs2 or due to the absence of a functional osmostress response. To be able to

differentiate between the two hypotheses the Sho1-Ste11 interaction was also analysed in a *Δhog1* strain (Fig. 17). Interestingly, this experiment showed a similar result to when a *Δpbs2* strain was used. Thus, the lack of Hog1-activation and therefore the lack of HOG signal transduction leads to an increased interaction rate of Ste11 and Sho1, indicating the presence of a negative feedback loop to shut down upstream components.

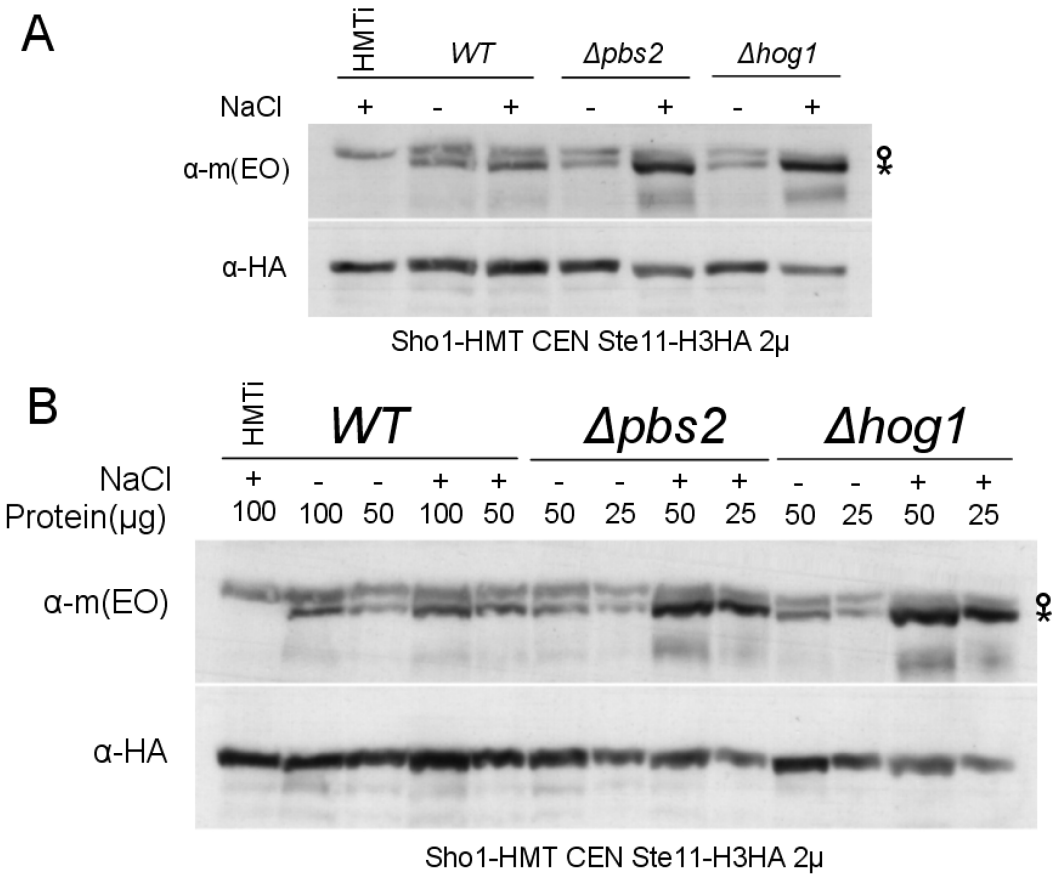


Figure 17: Interaction of Sho1-HMT CEN and Ste11-H3 2μ in *Δpbs2* and *Δhog1*. A Comparison of wild-type, *Δpbs2* and *Δhog1*. **B** Semi-quantification of the interaction in wild-type *Δpbs2* and *Δhog1*. For every sample two different protein concentrations were subjected to western analysis. * indicates the specific band, ° an unspecific band.

One possible explanation for these increased interactions of Sho1 and Ste11 in a *Δhog* or *Δpbs2* strain background is that the signalling cascade might become overactivated, because neither adaptation nor negative feedback happens in a Pbs2- or Hog1-deficient yeast cell. Furthermore, the catalytic activity of Pbs2 and/or the phosphorylation of Hog1 may be necessary to induce the dissociation of the signalling complex that includes Sho1 and Ste11. Therefore, the absence of Pbs2 or Hog1 may result in prolonged interaction of Ste11 and Sho1.

6.3.3 Ste11-Sho1 interaction depends on Ste50 but not on Opy2

Next we wanted to answer whether Sho1 and Ste11 can spontaneously bind to each other, or if other factors are involved that contribute to signal specificity. One factor that has been previously described to permanently interact with Ste11 is the adaptor protein Ste50. It has been previously published by Tatebayashi *et al*. (2006) that Ste11-Ste50 protein pair becomes recruited to the plasma membrane (and therefore to Sho1) during osmotic stress conditions. This is accomplished by the interaction of Ste50 with the membrane protein Opy2. To confirm this model, we analysed the interaction of Ste11 and Sho1 in *Δste50* and *Δopy2* strains. As expected, deletion of *STE50* caused the Sho1-Ste11 interaction signal to be strongly reduced (Fig. 18A, B). We observed a more than four times reduction of the interaction signals, when using semi-quantitative western analysis (Fig. 18B). However, in *Δopy2* cells the methylation signal did not change compared to wild type (Fig. 18B). This was surprising to us, because as mentioned before, Opy2 was described as being required for the recruitment of Ste50/Ste11 to the plasma membrane (Tatebayashi et al., 2006). However, repeated complementation analyses and osmosensitivity tests confirmed that the used strain is indeed *Δopy2*. These results indicate that Ste11 is targeted to Sho1 by Ste50, but this process is independent of Opy2.

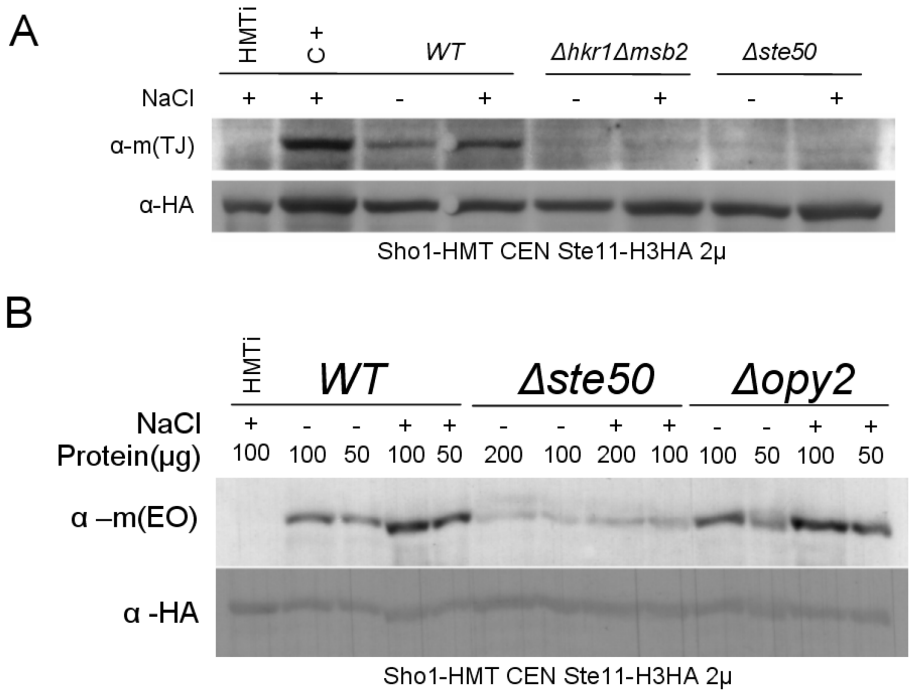
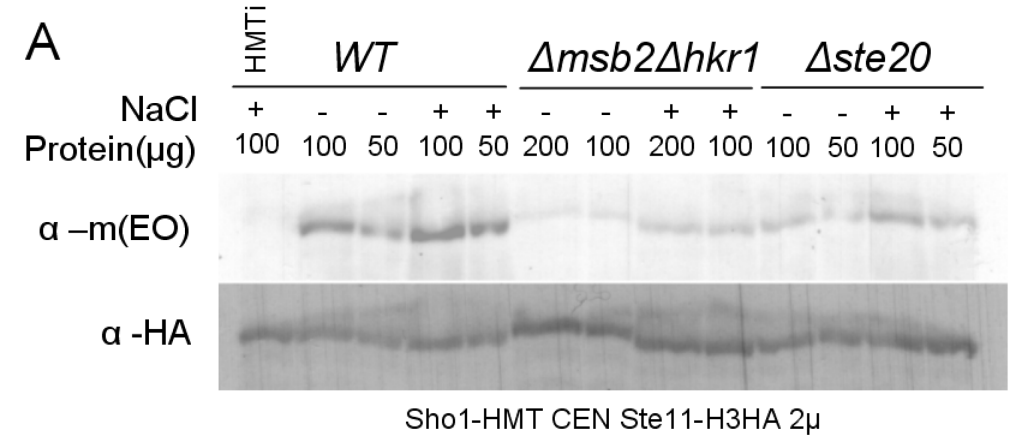


Figure 18: Interaction of Sho1-HMT CEN and Ste11-H3 2μ in *WT, Δmsb2Δhkr1*, *Δste50* and *Δopy2*. **A** Comparison wild-type, *Δhkr1Δmsb2* and *Δste50*; second lane shows a positive control (both construct expressed from a 2μ plasmid in wild-type). **B** Semi-quantification of the interaction in *Δste50* and *Δopy2* compared to wild-type. For every sample two different protein concentrations were subjected to western analysis. First lanes show inactive HMT as negative control.

6.3.4 The putative osmosensors Msb2/Hkr1 and Ste20 influence the interaction of Sho1 with Ste11

Since according to our observations, Opy2 is not necessary for Ste11 to be targeted to Sho1 and the plasma membrane, we wanted to test whether other membrane proteins of the SHO1 branch are involved in this process. To see if the putative osmosensors Msb2 and Hkr1 influence the binding of Ste11 to Sho1, this interaction was analysed in a *Δmsb2Δhrk1* double deletion strain (Fig. 18A, 19B). Interestingly, the Ste11-Sho1 interaction signal in this strain decreased by around 4-fold (Fig. 19A). Thus, Msb2 and Hkr1 have an impact on targeting Ste11 to the membrane and to Sho1. However, these results also show that there has to be some other mechanism inducing the increased interaction of Ste11 and Sho1, because the signals in the *Δmsb2Δhkr1* double knock-out strain are only reduced, but not abolished, and a signal increase of this interaction under osmostress can still be observed.



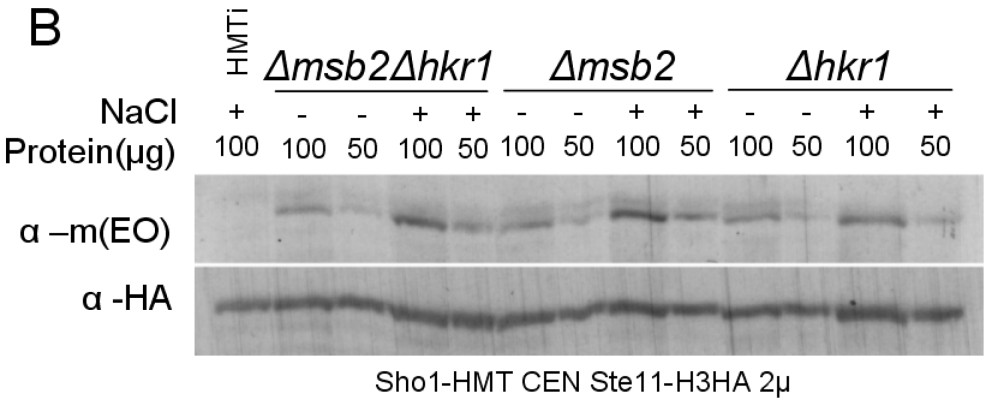


Figure 19: Semi-quantification of the interaction of Sho1-HMT and Ste11-H3. For every sample two different protein concentrations were subjected to western analysis. The first lanes show inactive HMT as negative control. **A** shows the comparison of this interaction in wild-type, *Δmsb2Δhkr1* and *Δste20* strains. **B** shows *Δmsb2Δhkr1* and *Δmsb2* and *Δhkr.*

We further analysed how the Ste11-Sho1 interaction would be affected in *Δmsb2* and *Δhkr1* single mutant strains. In both single mutant strains the Sho1-Ste11 interaction signal was similar to the signal generated in *Δmsb2Δhkr1* double deletion strains. This indicates that each individual single mutant compromises the Ste11-Sho1 interaction as much as the double mutant (Fig. 19B), and that both, Msb2 and Hkr1 are required for normal interaction levels.

We also investigated if the PAK-like kinase Ste20 influences the Ste11-Sho1 interaction. We analysed the interaction of Ste11 and Sho1 in a *Δste20* deletion strain (Fig. 19A) and discovered that the signal of this interaction was about 2-fold decreased in this strain. These findings indicate that all upstream components of the SHO1 branch are modulating the Ste11-Sho1 interaction.

6.3.5 The Sho1-Ste11 interaction depends on the severity of osmostress

To further analyse the Ste11-Sho1 interaction we performed a dosage response experiment. Wild-type cells containing Ste11-H3 and Sho1-HMT were treated with different salt concentrations varying between 0,2 and 1,4 M NaCl. As shown in figure 20 0,2 M NaCl hardly induced an increase of the Sho1-Ste11 interaction. However, gradually increasing the osmotic challenge to 0,8 M NaCl induced a faster accumulation of methylation signal: While at 0,4 M NaCl an increase in interaction could be seen only after 60 minutes (first blot, last lane), 20 minutes were sufficient to raise methylation signals at 0,8 M NaCl to similar levels (second blot, lane seven). Interestingly, higher salt concentrations delayed signal accumulation. While 1 M NaCl lead to increased methylation signals after 40 minutes (third blot, lane four), the addition of 1,2 M NaCl resulted in no increase in methylation over 60 minutes (third blot, lanes 6-9). This corresponds to earlier findings of Maeda *et al*. (1995) who discovered that the SHO1 branch of the HOG pathway needs at least 0,3 M NaCl to initiate a response and has a linear dose response up to about 0,6 M NaCl. However, one should remember that previously described experiments (Fig 7C) indicated that the methylation assay is sensitive to salt concentrations higher than 0,8 M NaCl. Therefore, the results obtained by this experiments performed at these concentrations have to be considered with care.

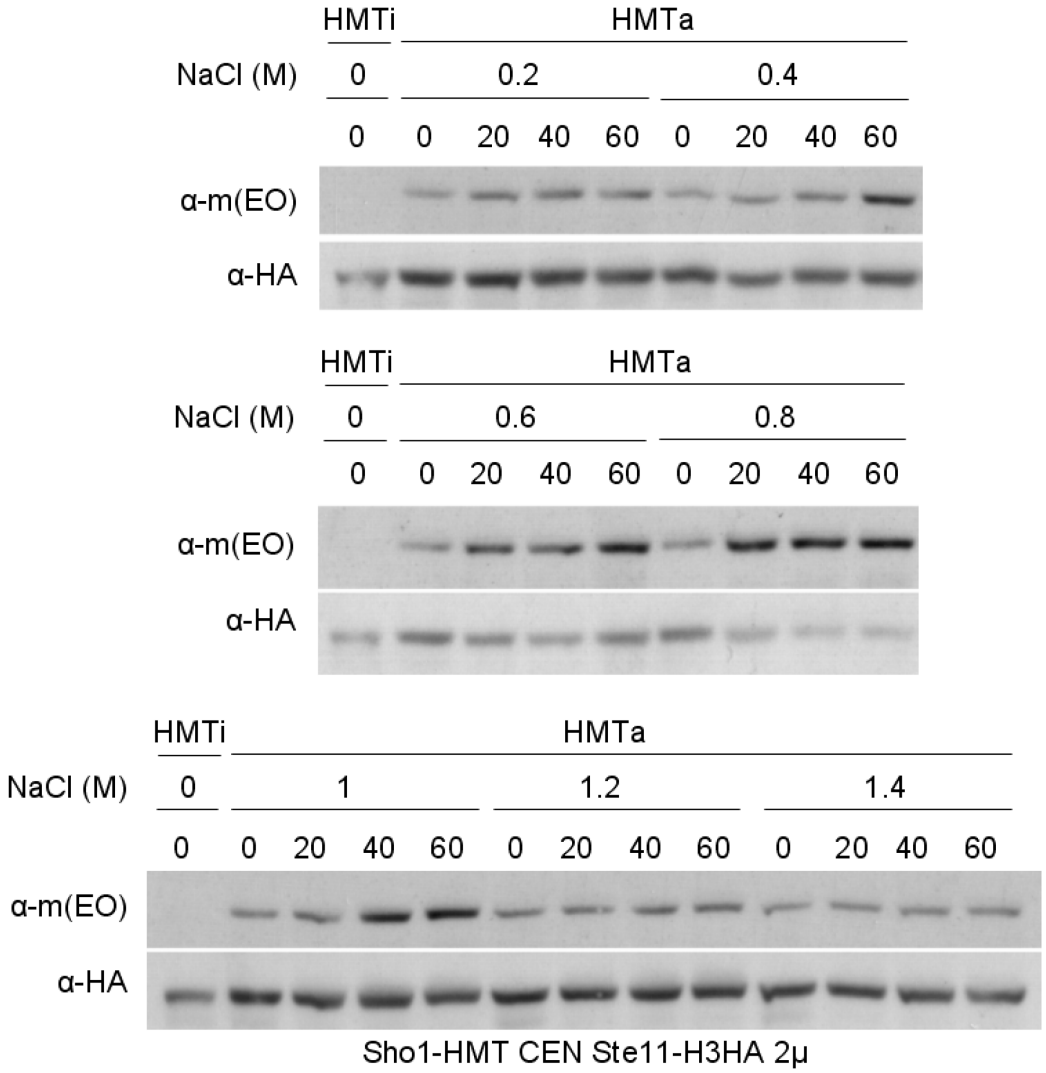


Figure 20: Dose response of the Sho1-Ste11 interaction. Co-expression of Sho1-HMT CEN and Ste11-H3 2µ in a wild-type strain. Salt stress was applied in concentrations from 0 – 1,4 M NaCl and samples were taken at indicated time points.

6.4 The many faces of Nbp2

As described in the introduction (chapter 3.1.2.).,one important way to regulate MAPK signalling cascades is negative feedback. These processes are required to down-regulate signalling cascades. Under non-stress conditions, they are required to keep basal signal transduction low. Furthermore, feedback loops have been described to possibly play a role in signalling specificity (Hao *et al*., 2008, Winkler *et al*., 2002). Often, negative feedback regulation of MAPK pathways requires phosphatases and phosphatase-associated proteins. As mentioned in the introduction, Nbp2 was shown to be a negative regulator of Hog1, possibly by recruiting the phosphatase Ptc1 to the Pbs2/Hog1 complex (Mapes *et al*., 2004).

We applied the methylation assay to investigate the Nbp2-Pbs2 interaction and specifically asked if we can possibly observe an interaction between these two proteins and if so, whether it is dependent on osmostress (Fig. 21). Interaction of Nbp2 and Pbs2 could be detected in both stressed and unstressed samples. However, this interaction was only slightly stronger in samples subjected to osmostress. Interestingly, this interaction was decreased stressed and unstressed protein samples in *Δste11* and *Δste20* single mutant strains, indicating that this interaction requires the presence of Ste11 and Ste20, or rather a fully functional SHO1 branch (Fig. 21A).

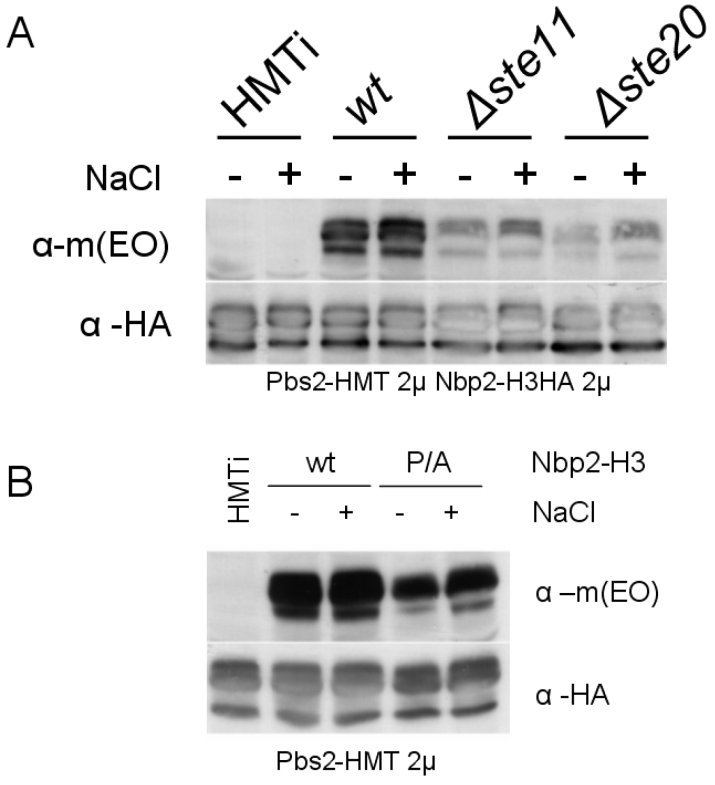


Figure 21: Interaction of Pbs2-HMT and Nbp2-H3, both expressed from a 2µ plasmid. **A** Comparison of wild-type, *Δste11* and *Δste20* strains **B** Co-expression of Pbs2-HMT with wild-type Nbp2 or Nbp2P/A-H3HA. Experiment was performed in a *Δnbp2* strain.

Next, we wanted to identify which domain of Nbp2 is required for recruitment to Pbs2. It has been previously observed that Nbp2 contains an SH3 domain. To analyse if this domain is of importance for this interaction, we substituted a conserved proline residue of the SH3 domain of Nbp2 with an alanine (P162A), which should in theory constitutively inactivate its binding capacity. Using this mutant we observed a significant decrease of the interaction of Nbp2 and Pbs2 (Fig. 21B). This finding supported the idea that Pbs2 and Nbp2 interact via the SH3 domain of Nbp2. However, this interaction signal was not completely abolished, suggesting that either this mutation does not fully inhibit the function of the SH3 domain, or that other motifs also play a role in the Npb2-Pbs2 interaction that are not affected by this mutation. Moreover, it is possible, that Nbp2 and Pbs2 do not only directly interact, but they may also associate via other factors of the SHO1 branch.

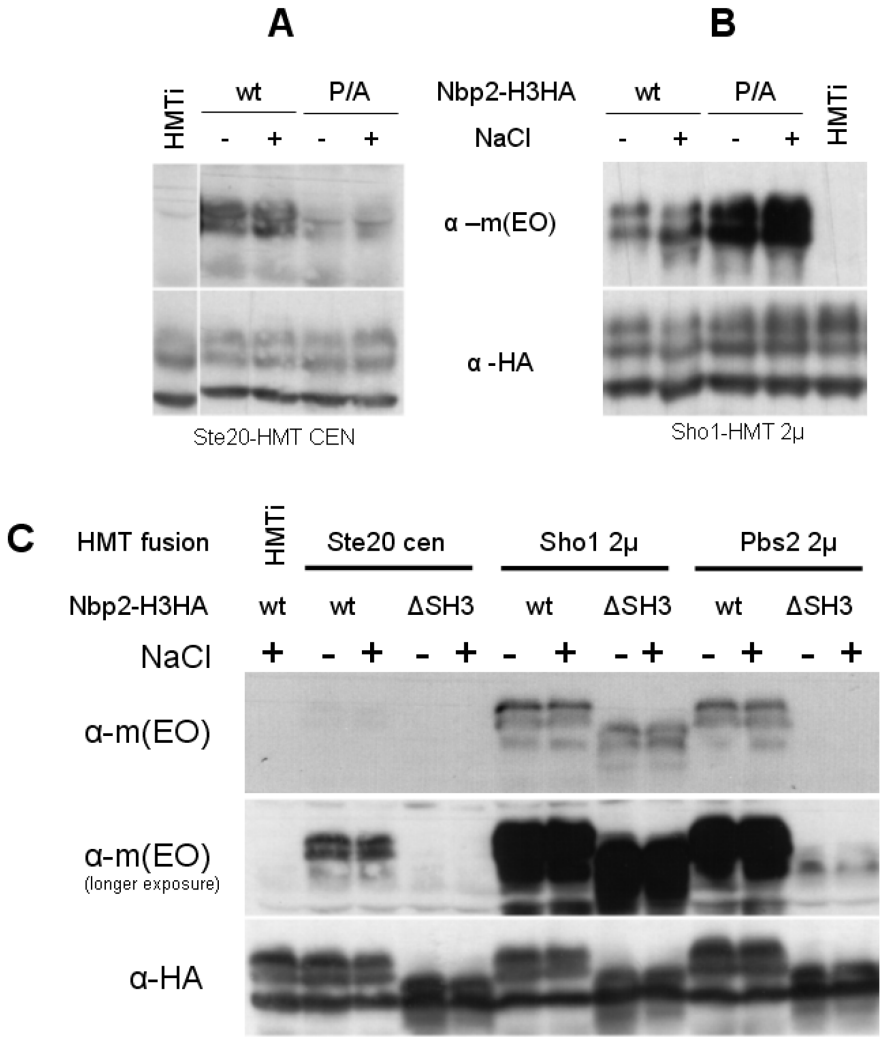


Figure 22: Impact of the Nbp2 SH3 domain on the interaction with different proteins. A Ste20-HMT CEN and Nbp2-H3 2µ wild-type and P/A mutant. **B** Sho1-HMT 2µ and Nbp2-H3 wild-type and P/A mutant. **C** Ste20-, Sho1- and Pbs2-HMT fusions assayed with Nbp2-H3 wild-type and ΔSH3. All experiments were performed in *Δnbp2*.

Furthermore, the interactions of Nbp2 with Ste20 and Sho1 were analysed, using the Nbp2 P/A allele. Consistent with the previous results and in line with our observations on the Nbp2-Pbs2 interaction, the interaction signal of Ste20 with the P/A mutant showed a decrease compared to wild type (Fig 22A). In contrast, the signal of the Sho1-Nbp2 P/A interaction increased compared to wild-type (Fig. 22B). This result was surprising, because rendering the SH3 domain non-functional should not result in increased binding to the signalling complex. Therefore, we next deleted the entire SH3 (Nbp2ΔSH3) domain of Nbp2 to analyse if the P/A mutation indeed mimics a non-functional domain or whether this mutation is still partially functional. Interaction of Nbp2ΔSH3 with Ste20 was completely abolished and also the interaction signal with Pbs2 was severely decreased. Conversely, Nbp2ΔSH3 and Sho1 showed no difference in methylation signal compared to wild-type Nbp2 (Fig. 22C). These results indicated that the interaction of Nbp2 with Ste20 and Pbs2 is dependent on the SH3 domain of Nbp2. However, Sho1 binding was not influenced by the deletion of the Nbp2 SH3 domain. Those experiments also showed that the P/A mutation does not render the SH3 domain completely non-functional since complete deletion decreased the methylation signal of the Nbp2-Ste20 or Pbs2 interaction even further. Moreover, the P/A mutant showed an increased interaction with Sho1, whereas complete deletion of the SH3 domain resulted in no changes in methylation signal compared to wild-type. This suggested that the P/A mutation may influence domains other than the SH3 domain as well, or may alter binding characteristics of the SH3 domain.

Next, we were interested to determine whether Nbp2 binds solely to Pbs2, or whether it also has affinity towards other proteins. Therefore, we investigated whether we could find interaction signal of Sho1 with Nbp2. Furthermore, since previous observations have indicated that the interaction of Pbs2 and Sho1 is dependent on the SH3 domain of Sho1, we figured that a Sho1ΔSH3 mutant should not be able to recruit a Pbs2-Nbp2 protein pair. However, if Nbp2 can bind Sho1 independently of Pbs2, the Sho1ΔSH3 mutant might still be able to produce an interaction signal with Nbp2. Additionally to the Sho1ΔSH3 mutant, we also looked at a series of internal deletion mutants of Sho1.

Indeed, we were able to detect interaction signals of Sho1 wild-type and ΔSH3, indicating that Nbp2 can be recruited to Sho1 independently of Pbs2. Moreover, different internal deletion mutants lead to a reduction of the interaction signal (Fig 23B). The strongest reduction was obtained with a Sho1Δ153-180 mutant. However, observed Nbp2 protein levels were decreased when this allele was used. In contrast, the Sho1Δ181-213

mutant showed slightly increased signals. These observations suggested that Sho1 interacts with Nbp2 via multiple sites of Sho1 rather than via a distinct binding motif.

We next wanted to investigate if the interaction of Sho1 and Npb2 is dependent on other components of the SHO1 branch. For this, we used a series of deletion strains to test, if the interaction of Sho1 and Npb2 becomes abolished or increased (Fig. 23A), indicating that this interaction is dependent on the respective gene product. In the wild-type control, an interaction signal for Nbp2 and Sho1 was clearly visible when cells were grown logarithmically and also when osmotic stress was applied. However, the signal was only slightly reduced in the *Δpbs2* mutant (Fig. 23A). Moreover, a much stronger reduction in methylation levels was found in *Δste11* and *Δste20* single mutant strains. However, it should be noted that Nbp2 protein levels also seemed to be slightly reduced in *Δste11* and *Δste20* strains. These findings contradict the previous observations by Ota *et al* (2007) who claimed that Nbp2 binds to the signalling complex via interaction with Pbs2. Furthermore, these findings indicated that the decreased interaction of Pbs2 and Nbp2 in *Δste11* or *Δste20* single mutant strains did not depend on the signal transduction of the SHO1 branch but rather on the two proteins themselves.

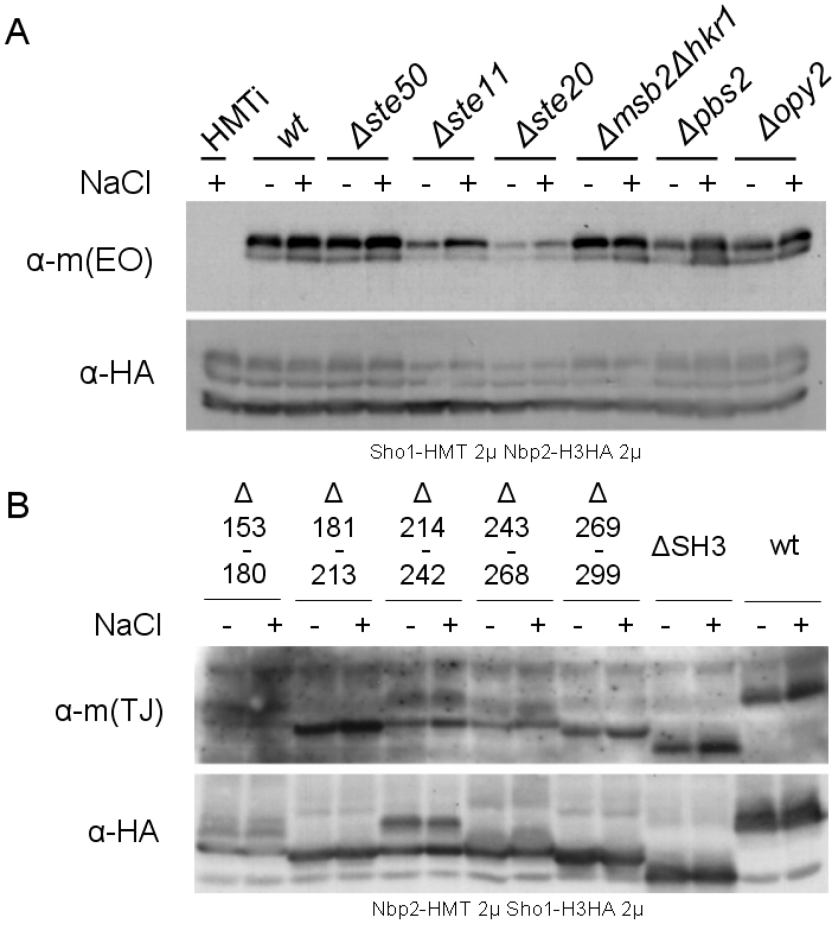


Figure 23: Interaction of Sho1 and Nbp2. A Expression of Sho1-HMT 2µ and Nbp2-H3 2µ in wild-type and different mutants. **B** Mutant series of Sho1-H3 2µ coexpressed with Nbp2-HMT 2µ.

While studying the interaction of Nbp2 with Sho1 and Pbs2 using the methylation assay we again encountered a problem: We could only detect methylation signals when expressing both proteins from 2µ plasmids. However, this may potentially distort results due to changed stochiometry of proteins. Therefore, it would be highly appreciated if methylation signals could be obtained under endogenous concentrations.

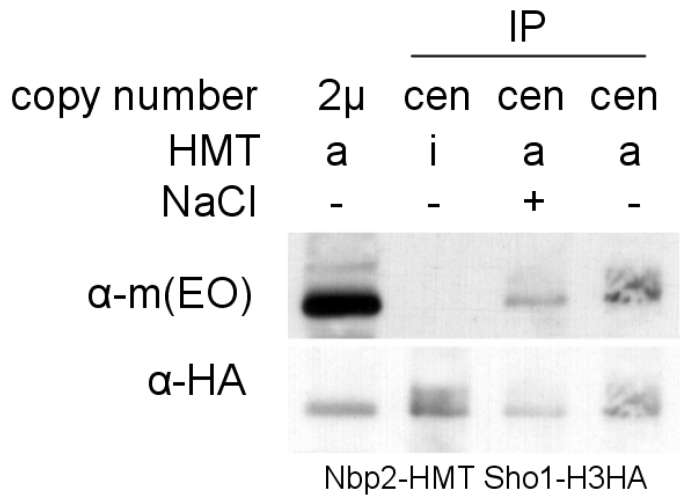


Figure 24: Analysing low abundant proteins. Co-expression of Nbp2-HMT and Sho1-H3 in *Δnbp2*. The first lane shows whole cell extract and interaction signal of Sho1-Nbp2 expressed from multicopy plasmids. Lanes 2-4 show an immunoprecipitation of Sho1-H3 expressed from centromeric plasmids

To verify that our observations are not based on overexpression artefacts but indeed represent true interactions, we performed an immunoprecipitation experiment with Sho1-H3 and Nbp2-HMT expressed from centromeric plasmids. α-HA coated Dynabeads (Invitrogen) were incubated with whole cell extract of cells expressing either the HMTa or HMTi. As seen in figure 24 we could indeed obtain methylation signals when adding an immunoprecipitation step. This result showed that PPIs can be detected under physiological concentrations with the methylation assay; however, an IP step may have to be applied additionally. Furthermore, this experiment confirmed the PPI of Sho1 and Nbp2.

To further investigate the function of Nbp2 and if it is indeed negatively regulating the HOG pathway at the level of Pbs2 or further upstream, a β-Gal assay was performed using a Fus1-LacZ reporter construct (Fig. 25). Using *Δpbs2* strains in combination with the application of osmostress leads to crosstalk and subsequent transcriptional activation of *FUS1*, a gene normally responsive to pheromone. Surprisingly, the reporter activation in a *Δnbp2Δpbs2* strain was significantly lower than in *Δpbs2* cells upon salt stress. This was in contrast to our expectancy. If Pbs2 would be negatively regulated, no changes in Fus1-LacZ expression should be expected. On the other hand, if upstream components like Ste11 or Ste20 would be negatively affected by Nbp2, the expectation would be that the Fus1-LacZ expression is increased in *Δnbp2 Δpbs2*. However, the obtained result fitted neither to one nor the other theory.

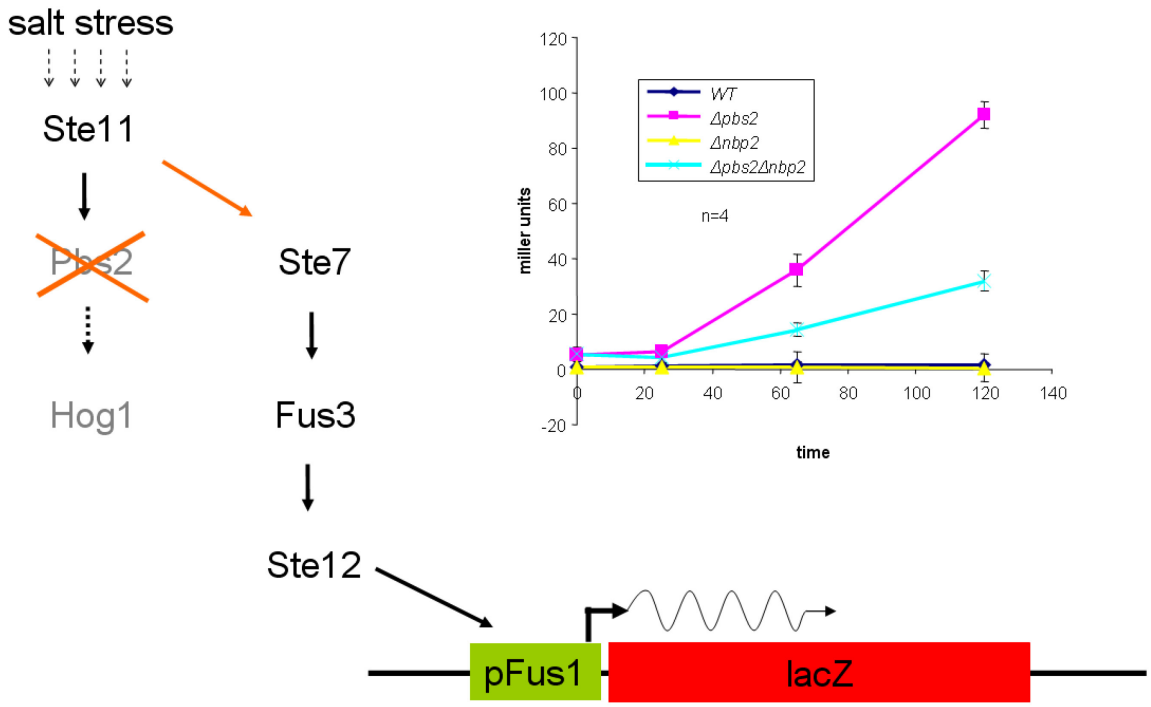


Figure 25: Fus1-LacZ reporter assay. A sketch of the cross talk leading to Fus1-LacZ expression by salt induction, and the impact Nbp2 has on it: Wild-type, *Δpbs2, Δnbp2* and *Δpbs2Δnbp2* stains were subjected to salt stress, and β-Gal assays were preformed in quadruplicates.

To rule out that this result was only caused by a side effect of the crosstalk and mislead pathway activation, an Stl1-LacZ reporter assay was performed. STL1 is a gene responsive to osmostress. This experiment was consistent with the results of the Fus1-LacZ reporter assay, showing reduced activity in the *Δnbp2* strain compared to wild type (Fig. 26). These experiments indicated that the role of Nbp2 in the HOG pathway is of more complex nature than of simply being a negative regulator. Its function in stress signalling might need to be reassessed in the future.

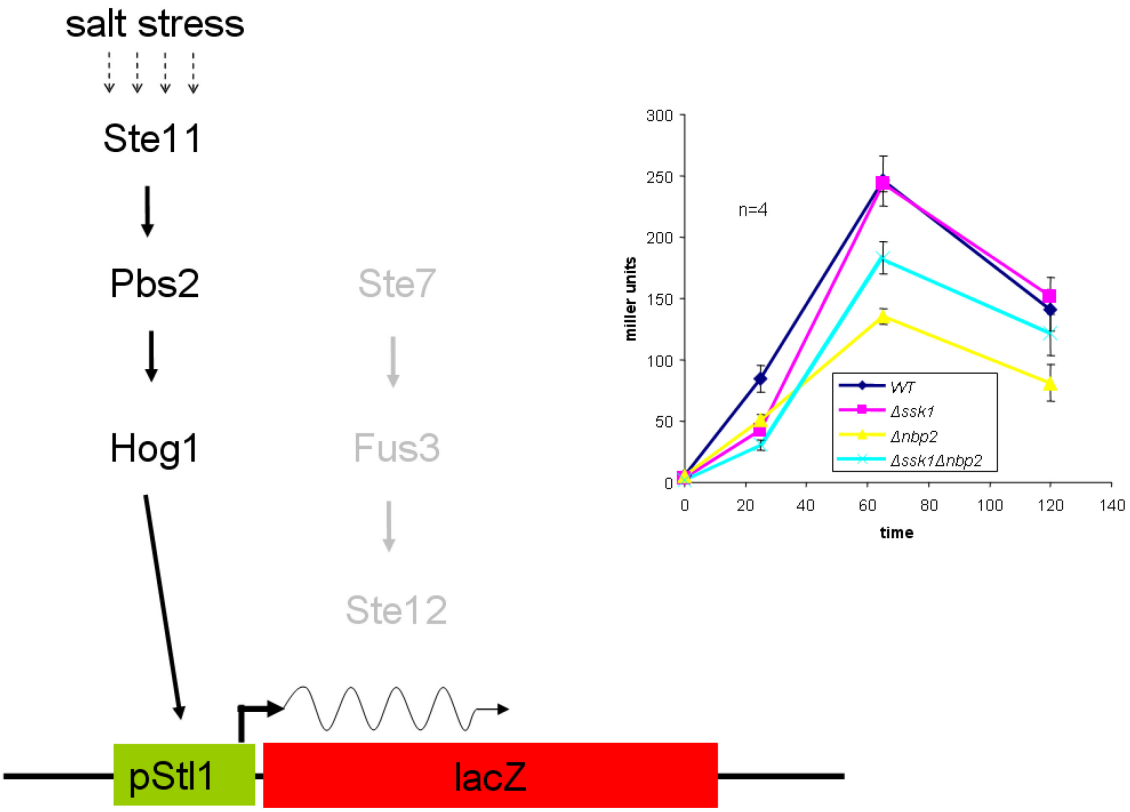


Figure 26: The impact of Nbp2 on Stl1-LacZ reporter construct expression: Expression of pStl1-LacZ in wild-type, *Δssk1, Δnbp2, and Δnbp2Δssk1* stains. Cells were subjected to salt stress and β-Gal assays were preformed in quadruplicates.

6.5 Fus1 interacts with Sho1 and negatively regulates the Sho-Pbs2 interaction

Fus1 is an SH3 domain containing protein that is poorly expressed during vegetative growth, but becomes induced upon pheromone response. Fus1 activity is necessary for cell fusion and it was suggested by Nelson *et al* (2004) to play a role in specifying signal transduction by competing with Pbs2 for Sho1 binding (Fig. 27A). Additionally to the SH3 domain Fus1 also contains a proline rich motif that was proposed to bind to the SH3 domain of Sho1. To prove this idea, Fus1 was tagged with the HMT and assayed for interaction with Sho1-H3 wild-type and the Sho1 affinity mutants Y8A and ΔSH3 (described in chapter 6.1.2.). Interaction signals could readily be detected under normal growth conditions (Fig. 27B). The Y8A affinity mutant of Sho1 showed reduced binding with Fus1, similar to the observations concerning the interaction of Sho1 and Pbs2. However, unlike the Pbs1-Sho1 interaction, deletion of the SH3 domain of Sho1 resulted in a strongly reduced, but not completely abolished methylation signal of Fus1-Sho1. This result showed that Fus1 binds Sho1 mainly, but not exclusively via the SH3 domain, making it a possible antagonist to Pbs2

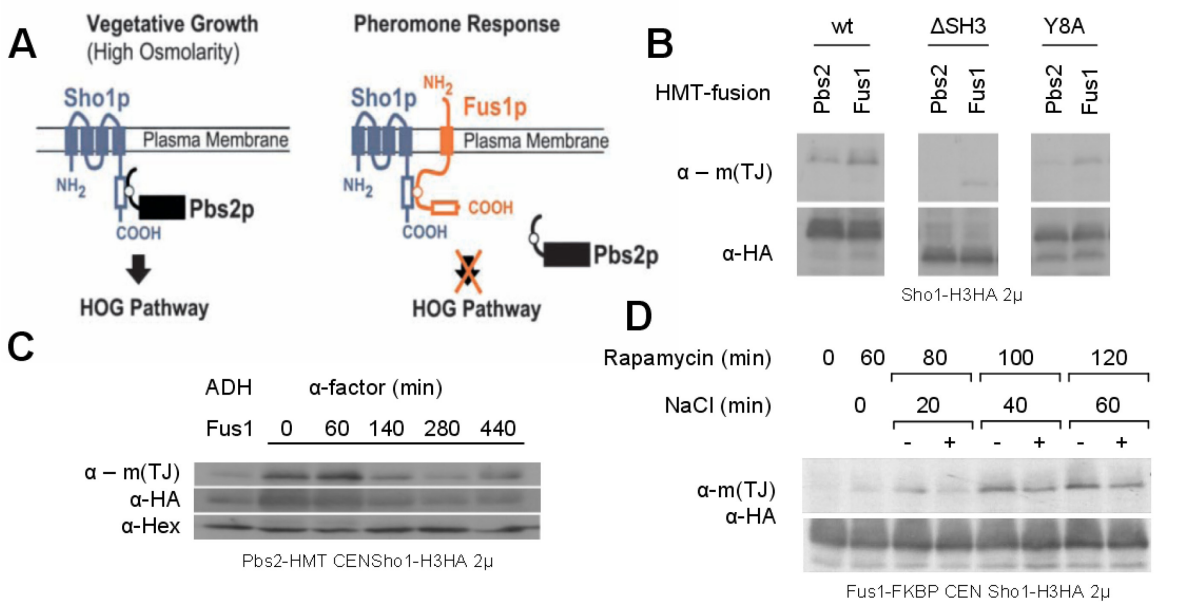


Figure 27: Interaction of Fus1 and Sho1. A A diagram from Nelson *et al*. (2004) how Sho1 and Fus1/Pbs2 may interact. **B** The interaction of Sho1-H3 2µ wild-type and mutants with Pbs2-HMT CEN or Fus1-HMT CEN in a *Δsho1* strain. **C** The interaction of Pbs2 and Sho1 in a wild-type strain in dependency of Fus1: Pbs2-HMT CEN and Sho1-H3 2µ were either coexpressed with Fus1 driven by the *ADH1* promoter (lane one) or the culture was treated with α-factor to induce endogenous Fus1 expression. **D** The interaction of *ADH1*-Fus1-FKBP CEN and Sho1-H3 2µ in Rapa^R using the conditional system (Co-expression with *ADH1* HMT-FRB): After pre-treatment of the culture with rapamycin for 60 minutes the sample was split and one half was subjected to 0,4 M salt stress.

To investigate this possible function of Fus1, we also analysed the Pbs2-Sho1 interaction of cells expressing Fus1 from the *ADH1* promoter during vegetative growth (Fig. 27C). Indeed, the Pbs2-Sho1 methylation signal was decreased when Fus1 was overexpressed compared to cells not expressing Fus1. Furthermore, the influence of pheromone treatment which leads to Fus1-expression was analysed. The interaction signal decreased over time in this experiment (Fig. 27C). Interestingly, Fus1 expression (due to addition of α-factor as well as constitutive Fus1 expression from the *ADH1* promoter) also resulted in the decrease of Sho1-HA signal, indicating that Fus1 may not only sequester Sho1 from Pbs2, but may also negatively affect Sho1 protein stability (Fig. 27C).

To investigate the behaviour of Fus1 during salt stress, we used the conditional methylation assay on the interaction of Sho1 and Fus1 (Fig. 27D). A similar signal pattern as for the interaction of Sho1 and Pbs2 was observed: Upon salt stress, the signal accumulates slower than without salt treatment representing a lower interaction rate of Fus1 and Sho1 upon pathway activation. This may be due to conformational changes of Sho1 upon its activation, leading to a decrease of the affinity of its SH3, independently of its current interaction partner.

7 Discussion

In this thesis a recently developed assay based on enzymatic tagging to study PPI in *S. cerevisiae* has been presented. The performance of this assay has been tested under different experimental conditions, fine-tuned and further developed. We applied this assay to address several open questions on the interplay of signalling components of the SHO1 branch of the HOG pathway. Although a number of components of this cascade has been previously identified and described, their interplay was not clearly resolved. Here, we present data showing that we were able to catch short-lived PPIs and transient changes in the affinities of proteins before and during activation of the HOG pathway. Furthermore, we investigated the recruitment of negative regulators contributing to feedback mechanisms. In addition, the dynamics of the interaction of Ste11 with Sho1 was investigated to analyse how this event can possibly regulate signalling specificity. We thereby contributed to a better understanding of the dynamic events taking place during SHO1 branch activation.

7.1 The methylation assay: Advantages and restrictions

Various methods to study signal transduction and PPI are available and are still being developed. However, every method has its own disadvantages and limitations (see introduction, chapter 3.2). The methylation assay presented here proved to be a new, extraordinarily useful tool to study PPI in budding yeast, expanding the palette of available methods in this field. We believe that the development of this described methylation assay has opened new possibilities in the analysis of protein interaction and signal transduction, which will be summarized now.

First, we have found the methylation assay to be a suitable means to follow dynamic interactions of membrane proteins: Membrane proteins have often been excluded from studies when other, previously established methods were applied. In strong contrast to most of these methods, we have experienced that especially PPIs between membrane proteins gave mostly strong methylation signals with the methylation assay. This phenomenon made them generally easy to be studied. We believe that these observed, strong intensities of signals may be due to the restricted diffusion of proteins in the membrane (in contrast to free diffusion in the cytoplasm).

Second, a very important advantage of the methylation assay is that we were able to catch transient interactions of proteins. For example, we were able to obtain signal differences for the PPI of Sho1 and Pbs2 or Sho1 and Ste11 (see chapter 6.1.2.3. and 6.3.). The interaction Sho1 and Pbs2 decreased upon stress treatment, representing lower interaction rates during pathway activation, whereas the interaction of Sho1 and Ste11 became increased upon stress treatment, representing higher interaction rates.

Third, a drawback of common methods is the high number of false positive and false negative results. With the methylation assay, the probability of gaining false negative results, caused by misfolding or unfavourable positioning of the tag (or the tagged protein) can be assumed to be roughly equal to other common methods that use protein tagging. However, we believe that the number of false positives should be lower than for many established methods, because the transfer of the methyl group appears to be not fast enough to produce a signal caused by transient interactions.

Fourth, our assay allowed us to study interactions of low abundant proteins under physiological conditions: In the work presented here, the main priority was to detect PPIs. Therefore, many interactions were studied using overexpression of one of both proteins from a 2µ plasmid. However, overexpression potentially influences cellular processes by altering the stochiometry of involved proteins. We showed that we were able to overcome the need for overexpression (and thus allowing to study PPIs at endogenous protein levels) when an immunoprecipitation step prior to western blotting was performed (see chapter 6.4). Lastly, no special equipment or expensive reagents are needed to perform the methylation assay.

Despite the just described advantages of the methylation assay, our newly developed assay does not overcome one problem common to all protein-protein interaction methods: It cannot distinguish between a direct and an indirect interaction; for example: When we performed the experiment depicted in Fig. 9A, we could observe that endogenous wild-type Sho1 was able to bridge the interaction of tagged Sho1ΔSH3-H3 and Pbs2-HMT. This is because Sho1 aggregates in homo-oligomers at the plasma membrane as it was previously described by Hao *et al*. (2007). In *Δsho1* cells those complexes solely contain Sho1ΔSH3-H3 which cannot bind to Pbs2 due to the lack of the SH3 domain. In contrast, in wild-type cells, endogenously expressed wild-type Sho1 forms heterogeneous complexes with the Sho1ΔSH3-H3 and Pbs2 can bind to those oligomers via the SH3 domain of wild-type Sho1. This way the proteins come in close vicinity and can transfer the methyl residue onto the H3 tag.

The second major problem we encountered was the variability of the used antibodies. The quality of western blotting as readout stands and falls with the reliability of the used antibody. No commercially available antibody suited our needs sufficiently. Unfortunately, the first type of antibody that we used (α-m(TJ)) lost its specificity. Therefore, during the course of this project a new antibody had to be generated (α-m(EO)), and the experimental conditions had to be re-optimized. We furthermore tested additional methylation antibodies for the following reason: In theory, the different canalisation rates of mono-, di- and tri-methylation could provide additional information about the transience of PPIs. However, the data presented here was mainly obtained by using α-tri-methylation antibodies. When mono- or di-methyl antibodies were used, we were not able to obtain reasonable results. However, these antibodies were only tested on the interaction of Sho1 and Pbs2. It is conceivable that maybe other protein pairs with different interaction dynamics could be analysed with these different antibodies.

To overcome at least some of the described problems we thought of including mass spectrometry as a readout system for future experiments. This technology will help to increase sensitivity, and will possibly also give further insights on the dynamics of the protein interactions, because it will allow us to differentiate between the three types of methylation. Furthermore, applying the methylation assay in combination with MS analysis may also overcome one drawback of the described method: Since in higher organisms endogenous H3K9 is methylated they are excluded from analysis of PPI with the methylation assay because this may cause severe background signals as well as potential interference of endogenous and artificially generated HMT and H3. However, HMT and H3 of higher eukaryotes are located in the nucleus (Firestein *et al*., 2000). Therefore, particularly plasma membrane proteins may still be studied, especially if the methylation assay is applied in combination with IP and MS.

7.2 The interactions of proteins at the plasma membrane in the SHO1 branch:

7.2.1 The interaction of Sho1 and Ste11 is a crucial step in osmostress signalling

The crucial step in the activation of the Hog1 MAPK by SHO1 branch signalling is that the MAPKKK Ste11 becomes sequestered by Sho1 and specifically acts on the MAPKK Pbs2,

and does not phosphorylate any of the other MAPKKs. We applied our methylation assay to study this activation step and addressed the following three questions: How do the interaction affinities of Ste11 and Sho1 change during osmostress? What upstream and downstream components of the SHO1 branch influence this reaction? How does this step contribute to signalling specificity (fig. 28)?

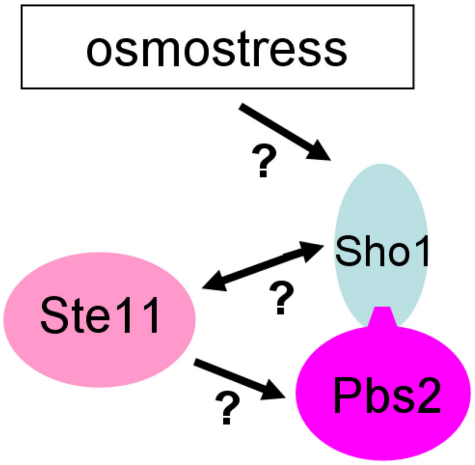


Figure 28: the Sho1-Ste11-Pbs2 signalling module. How does the interaction of Sho1 and Ste11 change upon osmostress, what factors alter this interaction and how does this step contribute to signalling specificity?

7.2.1.1 The increased interaction rate between Ste11 and Sho1 functions only in a limited concentration window

Our experiments showed that Ste11 interacts with Sho1 during basal growth conditions and that this interaction becomes enhanced during osmostress (see chapter 6.3.). Besides, we also could observe that the Sho1-Ste11 interaction is influenced by the severity of osmostress. We observed that the SHO1 branch efficiently responds to concentrations of NaCl between 0,4 and 1 M (chapter 6.3.5.). This observation correlates well with data from Maeda *et al.* (1995) and van Wuytswinkel *et al.* (2000) who showed that in an SLN1 branch deficient strain (*Δssk2Δssk22*) Hog1 becomes activated at NaCl concentrations between 0,3 M NaCl and 1,4 M NaCl. Our observations indicate that the inability of Sho1 to increase its interaction rate with Ste11 at lower or higher concentrations of NaCl leads to the inability of the SHO1 branch to respond. This is probably due to an altered behaviour of Sho1 at low or high salt concentrations (rather than an altered behaviour of Ste11). We speculate that a different glycosylation state of the Sho1/Msb2/Hkr1 sensing complex at high salt concentrations reduces Sho1's affinity to Ste11 (reviewed in de Nadal *et al.*, 2007).

7.2.1.2 The Ste11-Sho1 interaction is influenced by up- and downstream components

We and others have shown that different upstream and downstream components have an impact on the interaction of Sho1 and Ste11: Whereas deletion mutants of the upstream components Hkr1/Msb2, Ste20 and Ste50 resulted in reduced interaction of Ste11 and Sho1, deletion of the downstream components Pbs2 and Hog1 resulted in enhanced interaction upon stress treatment. Interestingly, the interaction did not change in a *Δopy2* strain background, which was previously described as a potential upstream component of the SHO1 branch.

The observed decreased interaction of Ste11 and Sho1 in a strain deleted for the putative osmosensors Msb2 and Hkr1 may reflect that Sho1 does not become fully activated, because of reduced upstream signal input leading to a decreased recruitment of Ste11 in this strain background. Also, the deletion of *STE20* showed a decreased interaction of Ste11 and Sho1; however, this effect was less severe than in a *Δste50* or a *Δmsb2Δhkr1* background. Furthermore, we have shown that Ste50 has an important role in targeting Ste11 to Sho1. Deletion of Ste50 resulted in a significant reduction in the interaction of Ste11 and Sho1 (see fig. 18). This finding is consistent with previous observations that Ste50 is essential for SHO1 branch signalling (Posas *et al*., 1998). Its main responsibility for SHO1 signalling lies in the recruitment of Ste11 to the plasma membrane (and therefore to Sho1). Moreover, it has been reported that Opy2 contributes to the Ste50 dependent recruitment of Ste11 to the plasma membrane (Wu *et al*., 2006). However, as mentioned above, we have not been able to detect an influence of Opy2 on the Sho1-Ste11 interaction (see fig. 18).

Additionally, our experiments clearly indicated that the components of the SHO1 branch do have a basal affinity to each other, independent of osmostress: First, we obtained methylation signals without stress application; second, the reduced signals of the Sho1-Ste11 interaction in upstream signalling incompetent strains (*Δmsb2Δhkr1 Δste20* and *Δste50*) show that this affinity is independent of osmostress.

7.2.1.3 Opy2 does not influence the affinity of Ste11 and Sho1

It has been a matter of intense research and speculation how proteins like Ste11, Ste20 and Cdc42 manage signalling specificity, despite being signalling components shared by different pathways. Studying this problem is particularly hard, because it is almost certain,

that not all molecules of one type of protein will react in the same way to a given condition. Rather, only a subset of them will become activated under a given condition and will then transfer the signal exclusively to the right downstream target and thereby prevent crosstalk. Furthermore, all MAPK signalling cascades in yeast may theoretically be slightly active below a certain threshold level at any given time. Therefore, in order to survive and propagate, a cell has to tightly guard proteins that function in different pathways. Wrong signalling of e.g. Ste11 would be fatal since it could lead to the activation of three individual pathways. It has been speculated that one way a cell guarantees specificity of signal transduction is that different types of protein complexes, each specific for its individual pathway, are formed. This way it may be that only a fraction of a cell's Ste11 molecules (that is part of the correct signalling complex) becomes activated upon a certain condition. Another possibility would be that complexes don't exist prior to activation but that only components specific for the activated pathway sequester active Ste11.

Interestingly, we observed that almost all tested PPIs of components of the SHO1-branch gave a signal above background independently of stress treatment (see for example fig. 14). This observation can be interpreted in three different ways: First, indeed the components of the SHO1-branch are permanently sequestered and thereby possibly guarantee signalling specificity. Second, the MAPK cascade may permanently be slightly activated by minor osmotic perturbations. Third, our assay responds to fortuitous interaction events.

As mentioned before, methylation rates of our assay correlate with published binding affinities. Additionally, prevention of interaction of two proteins resulted in complete loss of methylation signal (Fig. 9). Therefore, we strongly believe that our assay does not catch random but specific PPIs. Furthermore, as shown in figure 20 and discussed in chapter 6.3.5., SHO1-branch activation requires a concentration above a certain threshold, which strongly argues against a permanent activation of the cascade. Therefore our data support the idea that some components of the SHO1-branch are permanently assembled in at least a loose association. However, for most of the studied PPIs we detected a significant increase in interaction. Therefore both, loosely preassembled proteins and specific recruitment in combination contribute to specific activation and signalling specificity.

Our model is a combination of the presented ideas: During vegetative growth, Ste11 interacts with a low affinity with all it potential binding partners. Upon activation of the HOG pathway, the interaction rate of a HOG determined subpopulation of Ste11 increases

to Sho1. Thereby, the interaction frequency of dynamic PPI to those pathway specific components specifies the signal transduction. A similar mechanism with a different subpopulation of Ste11 may be employed for the activation of the FG pathway. We speculated that Pbs2, the HOG specific MAPKK activated by Ste11, contributes to differentiation between the HOG and the FG pathway. Additionally, we wanted to investigate, how Pbs2, Ste50 and Opy2 possibly contribute to the definition of the subpopulations of Ste11.

As already mentioned before, the interaction of Sho1 and Ste11 is not dependent on Opy2. This was surprising, because recent publications have described Opy2 to be an essential protein for SHO1 branch signalling, interacting with Ste50 and therefore recruiting Ste11 to the signalling complex (Wu *et al*., 2006; Tatebayashi *et al*.,2006; Ekiel *et al*., 2009). The studies of Tatebayashi et al. (2006) were based on the interaction experiments of Ste50 and Opy2. We were able to detect PPI of Ste50 and Opy2, and this interaction was not significantly altered when using the Ste50D/F mutant. However, this mutant was shown to be constitutively recruited to the membrane and was able to induce the HOG pathway in combination with the constitutively active Ste11Q/P allele (Tatebayashi *et al*.,2006). A possible explanation for our results together with these recent publications is that Opy2 may not influence the affinity of Sho1 and Ste11/Ste50, but rather influences the correct positioning of interacting signalling components towards each other. The recruitment of Ste11 to Sho1 may be independent of Opy2; however, Opy2 may be required to correctly orientate Ste11 and Sho1 so that signal transduction becomes possible. Another explanation for how our results fit together with the fact that Opy2 is essential for SHO1 signalling is the following: Opy2 was originally described to be able to suppress a pheromone induced cell cycle arrest as an overexpression mutant. This observation might be a hint that Opy2 is only indirectly required for SHO1 branch signalling. Opy2 may not bind the fraction of Ste50 molecules that act in SHO1 branch signalling, but maybe a fraction of Ste50 that activates the pheromone pathway. An explanation for why after all Opy2 is essential for the SHO1 branch, although it does not directly act on it would be that an active pheromone pathway possibly suppresses the HOG cascade (possibly by the high turnover of Ste11 during pheromone treatment, Esch *et al*., 2002)(Fig. 29).

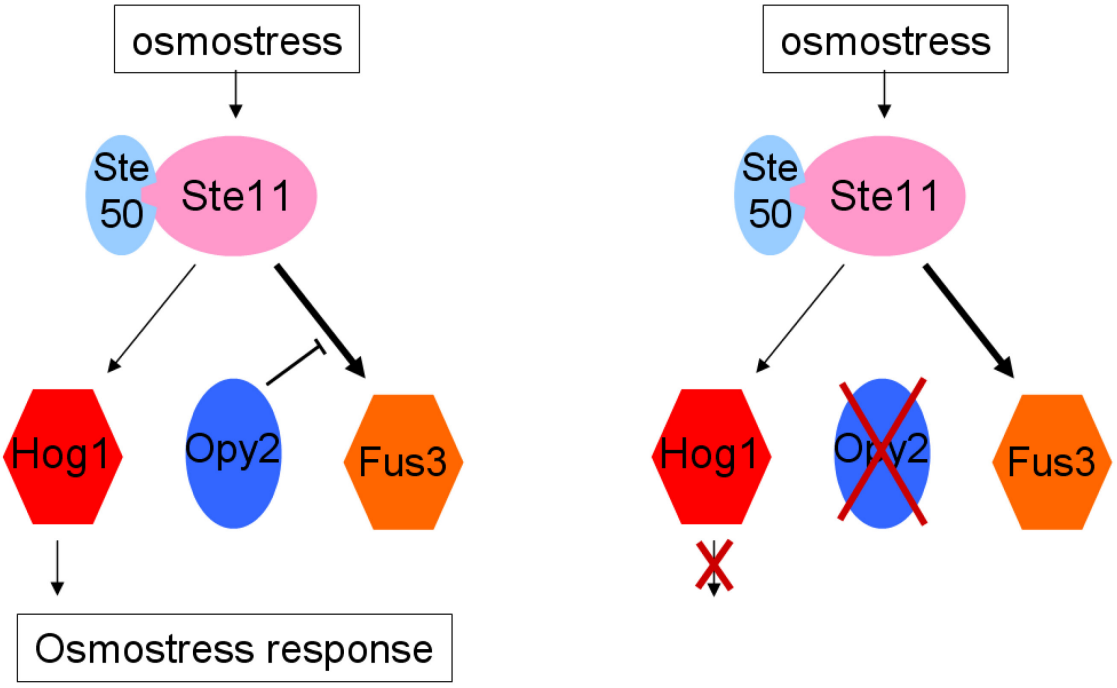


Figure 29: Opy2 may contribute to HOG signalling specificity in an indirect manner: During osmostress response, Opy2 may inhibit the signal transduction of Ste11 to the mating response. If Opy2 is deleted, Ste11 may preferentially signal via the pheromone pathway, and in turn inhibit the HOG pathway.

7.2.1.4 Ste50 contributes to Sho1 signalling specificity in a direct manner

To investigate the membrane recruitment of Ste11 in more detail, and to determine, how this step may contribute to signalling specificity, we tested the Sho1-Ste11 interaction in Ste50 mutants that showed different binding characteristics. Investigations of Tatebayashi *et al* (2006) identified a mutant Ste50 allele (D/F) that could activate Hog1 in combination with a constitutively active Ste11 allele (Q/P) without stress application. In contrast to wild-type Ste50, Ste50D/F did co-precipitate with Sho1. However, we obtained slightly less interaction signal of Ste50D/F with Sho1 (and also with Cdc42) compared to wild-type, indicating that the D/F mutant is not constitutively recruited to Sho1. Furthermore, the D/F mutant did not show any difference in the methylation signal with Opy2 compared to wild-type Ste50. Therefore, we were not able to confirm that Ste50D/F permanently recruits Ste11 to the membrane. This suggests that the different properties of the two applied methods, methylation assay and Co-IP, could be responsible for the contradicting results that we have obtained. The Co-IPs were performed in a *Δsho1Δste11Δste50Δpbs2* strain whereas the methylation experiments were performed in a *Δste50* strain. Deleting several members of the signalling complex may lead to aberrant binding affinities of Ste50. While the binding of the Ste50D/F mutant allele to Sho1 may be increased when examining only those two proteins (as it has been done in the Co-IP experiments), the interaction may be

decreased in the endogenous protein context, where proteins can compete with each other, as it was shown with the methylation assay. Therefore, no definite conclusions could be drawn and further analysis using the different Ste50 alleles will be required.

But how does Ste50 come into play? We and others have shown that Ste50 directly targets Ste11 to the plasma membrane (Wu *et al*., 2006, Truckses *et al*., 2006). We also could track PPIs of Ste50 with Opy2 and Cdc42, and the interactions with the latter two were strongly decreased in the Ste50P/L mutant. Moreover, binding of Ste11 and Sho1 strongly depended on Ste50, but neither the D/F nor the P/L mutation greatly altered the interaction signals of Ste50 and Sho1. This indicates that Ste50 binds Sho1 and Cdc42/Opy2 with two separate binding domains. These different domains may be individually regulated in response to different stimuli and may therefore play an important role in signalling specificity. Furthermore, in a recent publication it has been speculated that although Ste11 and Ste50 bind each other constitutively, there might be different complex species of this protein pair present within the cell (Slaughter *et al*., 2008). Therefore, Ste50 may have an additional regulatory role besides the recruitment of Ste11.

7.2.2 Active Pbs2 enhances dissociation of the Sho1-Pbs2-Ste11 complex

Pbs2 is known as a versatile protein with two major functions. First, it encodes the MAPKK of the HOG pathway. Second, it is also thought to act as scaffold protein for the HOG pathway. In contrast to many other proteins of this pathway, Pbs2 seems to interact with Sho1 solely via a proline rich motif that binds the SH3 domain of Sho1. We have been able to observe that the methylation signal of the Sho1-Pbs2 interaction becomes completely abolished in a Sho1ΔSH3 mutant. This finding indicates that if this interaction is disabled, Pbs2 becomes completely separated from the signalling complex. Other protein interactions showed only strongly reduced, but not completely abolished signals, suggesting that a certain affinity remains possibly due to other PPIs within the signalling complex (see for example chapter 6.3.5.). Our observation indicates that there is no additional interaction domain influencing the affinity of Sho1 and Pbs2. Therefore, Pbs2 is attached to the SHO1 signalling complex by directly binding to Sho1. Interactions of Pbs2 with Ste11 or possibly also with other proteins may be assured via the action of Sho1.

With our work we support a novel idea, that in addition to its function as a kinase and as scaffolding protein, active Pbs2 is required for the dissociation of the Sho1-Pbs2-Ste11 complex. We could observe a constitutive interaction between Sho1 and Pbs2

independent of pathway activation. The interaction rate between the proteins became decreased upon stress signalling, indicating that the complex collapses upon its activation. We also showed that the interaction of Ste11 and Sho1 was strongly increased in *Δpbs2* and *Δhog1* strains upon pathway activation indicating that a functional signal transduction cascade is necessary to separate the components of the signalling complex.

Furthermore, microscopy-studies didn't reveal the localization of wild type Pbs2 or Ste11. Our data suggests that it may be very likely that Pbs2, Ste11 and Sho1 constitutively associate and dissociate at the plasma membrane. Upon pathway activation Sho1 increases the on-rates of Ste11, which probably leads to the subsequent phosphorylation of Pbs2. Only upon activation of Hog1 by Pbs2 the off-rate of Pbs2 is increased, resulting in the dissociation of the signalling complex. Finally, the prolonged interaction of Ste11 and Sho1 in *Δpbs2* or *Δhog1* cells may represent a mechanism of signalling specificity: To avoid aberrant signal transduction Sho1 tethers Ste11, thereby forcing it to act on Pbs2 and not on any other MAPKK. Only upon Hog1 activation Ste11 is released from the signalling complex (fig. 30).

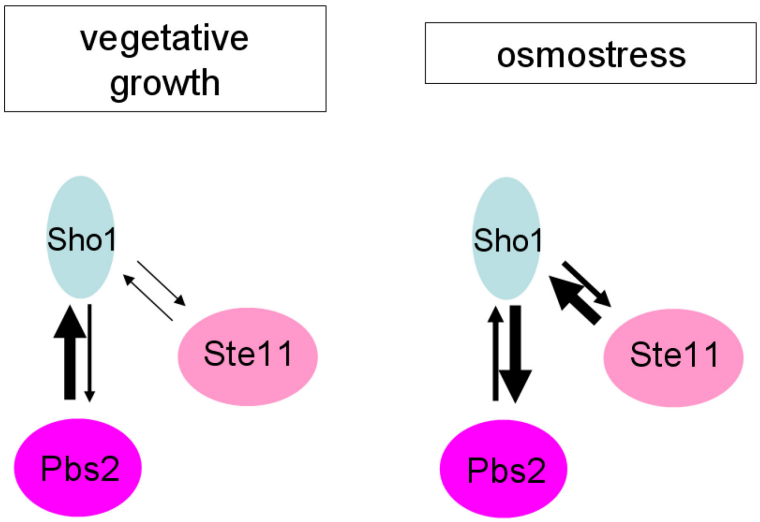


Figure 30: Association and dissociation of the Sho1-Ste11-Pbs2 signalling complex. During vegetative growth, Sho1 and Pbs2 strongly interact, whereas the interaction of Sho1 and Ste11 is loose. Upon osmostress the on-rate of Ste11 and Sho1 increases, leading to the activation of Pbs2. Subsequently, the off-rate of Pbs2 and Sho1 increases leading to the dissociation of the complex.

7.2.3 Rtn2 shows reduced interaction with Ste20 upon osmostress

Previously, Rtn2 has been annotated with shaping and bending of membranes. However, recently Rtn2 was also found to be of importance for osmostress signalling in a screen performed by V. Reiser and S. Salah (unpublished data). In the work presented here we have discovered that Rtn2 is able to interact with several components of the SHO1 branch. Furthermore, we found Rtn2 protein levels to become increased during salt stress conditions, which is due to transcriptional activation of the *RTN2* gene by Hog1,

confirming that Rtn2 plays a role in osmostress adaptation. However, based on our observations, the function of Rtn2 has become not only related to osmostress, but has specifically been connected to the SHO1 branch of the HOG pathway. Rtn2 has been found to be essential for this branch because *Δssk1Δrtn2* strains were shown to be osmosensitive. Additionally, this mutant strain exhibits impaired localisation of the Pbs2K/M mutant, an allele that is catalytically inactive and was shown by GFP studies to co-localise with Sho1 upon osmostress (S. Salah, V. Reiser, unpublished data). However, we speculate that the most important function of Rtn2 in stress signalling lies in its interaction with Ste20 (therefore Rtn2 may only indirectly influence the localisation of Pbs2): Our results showed that only the interaction rates of Ste20 and Rtn2 changed upon osmostress. Therefore, we believe that this interaction might be essential for the activation of the SHO1 branch. Furthermore, this observation is in line with a localization study of Pbs2K/M: This mutant allele co-localized with Sho1 only upon osmostress treatment. However, if Rtn2 is absent, upstream signalling at the level of Ste20 will be inhibited, and this will lead to a decreased activation of the signalling cascade. In such a situation, Pbs2 cannot be activated efficiently. Therefore, the Pbs2K/M allele will not localize to sites of polarised cell growth in a *Δrtn2* strain background under osmostress conditions.

The exact function of Rtn2 however remains elusive: It plays dual role in bending membranes on one hand, and allowing signal transduction via the SHO1 branch on the other hand. However, since osmostress has a large impact on the plasma membrane, we speculate that Rtn2 may work in sensing changes in the plasma membrane.

7.2.4 Fus1 sequesters Sho1 to inhibit false signalling and to promote mating

Previously it has been shown that Fus1 competes with Pbs2 for binding site on Sho1 (Nelson *et al*. 2004). Therefore, it has been speculated that Fus1 is involved in inhibiting crosstalk between the mating and the HOG pathway (Nelson *et al*. 2004). With the data presented here (chapter 6.5.) we confirmed this finding, and moreover discovered that Fus1 also regulates protein levels of Sho1. Therefore, based on the observation that Fus1 and Sho1 are able to interact, we believe that it is more likely that Fus1 affects Sho1 protein stability rather than Sho1 protein synthesis. We speculate that a cell that undergoes mating might benefit from a reduced abundance of Sho1. That way, a larger fraction of a cell's Ste11 molecules becomes accessible for the mating response pathway. Upon osmostress Fus1 is absent from the cell, and can therefore not interfere with HOG pathway signalling.

Our data provide an explanation for the higher affinity of Sho1 towards Fus1 compared to the interaction of Sho1 and Pbs2: Interaction of Fus2 and Sho1 seems to be supported by an additional binding domain besides Sho1's SH3 domain. We observed that the interaction signal of Fus1 and Sho1 becomes reduced, but not completely abolished, when a deletion of the SH3 domain is used. In contrast, the interaction signal of Pbs2 and Sho1 was completely abolished when using this mutant.

We could also show that the Fus1-Sho1 interaction, similar to the Pbs2-Sho1 interaction, decreases upon salt stress. This observation indicates that Sho1 undergoes a conformational change upon osmostress and thereby changes the binding parameters of the SH3 domain towards its binding partner. Apparently, this change in affinity is independent of the interaction partner.

7.3 Nbp2 is a positive and a negative regulator of the HOG pathway

After adaptation to a certain stress condition, cells need to be able to shut down the respective MAPK signalling. Therefore, negative feedback of the induced pathway is of high importance for survival. This negative regulation is often ensured by phosphatases and their associated proteins. Nbp2 has been described to be a negative regulator of the HOG pathway by being an adaptor for phosphatases. It has been shown to recruit Ptc1 to the signalling cascade (Mapes *et al*., 2004), leading to dephosphorylation of Hog1. Indeed we could detect interaction signals of Nbp2 with the proposed binding partner Pbs2. However, our results suggest a more complex picture of the function of Nbp2.

Unfortunately, our analysis of Nbp2 binding preferences and modes left many questions unanswered: We were able to show that the deletion of the SH3 domain of Nbp2 significantly decreased the interaction signal of Nbp2 with Ste20 and Pbs2. Mutating a conserved proline of the SH3 domain of Nbp2 (Nbp2P/A) led to similar observations, even though the decrease of the signal was not as severe as in the ΔSH3 mutant. In contrast, deletion of the SH3 domain of Nbp2 did not alter the Sho1-Nbp2 interaction and interestingly, the Nbp2P/A mutant gave even stronger methylation signals. This indicates that the P/A mutation does not corrupt, but rather changes the binding parameters of the SH3 domain. Furthermore, Nbp2 may bind to different signalling complexes via different binding motifs: On one hand Nbp2 binds to a complex containing Ste20 and Pbs2 mainly via its SH3 domain. On the other hand Nbp2 interacts with Sho1 independently of the SH3 domain of Nbp2. Nevertheless, we were not able to narrow down the region on Sho1, where Nbp2 binds preferentially. A deletion series of Sho1 did not provide us with a

pronounced region for Nbp2 binding. Also, deletion of the SH3 domain of Sho1 did not show any changes in the interaction signal of Sho1 and Nbp2, indicating that the Sho1-Nbp2 interaction is largely independent of Pbs2.

One role of Nbp2 may be to keep the basal signal of the HOG pathway low by constantly interacting with Pbs2 and therefore recruiting Ptc1. In *Δste11* and *Δste20* strains, no signal is transmitted via the SHO1 branch, and the affinity of Nbp2 to Pbs2 and Sho1 becomes reduced, suggesting a negative feedback loop. On the other hand, deletion of other essential components of the SHO1 branch did not lead to reduced interaction of Sho1 and Nbp2, indicating that functional signal transduction via the SHO1 branch is not required for the recruitment of Nbp2. Furthermore, the finding that the Sho1-Nbp2 interaction is only inhibited by the upstream kinases made us speculate that this recruitment is independent on localisation and functionality of signalling, but only depends on the activity of the upstream kinase proteins and therefore not on Pbs2 and its phosphorylation status.

Our experiments indicated that Nbp2 may also have a direct or indirect positive influence on the HOG pathway. A Fus1-LacZ reporter assay in a crosstalk competent strain (*Δpbs2*) showed a decreased reporter activity in the *Δnbp2Δpbs2* double mutant when compared to a *Δpbs2* single mutant. Corresponding results were obtained using a Stl1-LacZ reporter construct that is directly activated by the HOG pathway. Although this result was surprising at the beginning, this is an indication that Nbp2 has multiple functions: On one hand it is a negative regulator of Hog1, on the other hand, it may supply a positive regulatory function for the HOG pathway by negatively regulating other MAPK pathways (fig. 31).

Npb2 has been previously described to play an essential role in the cell wall integrity pathway and it was proposed that the CWI and HOG pathway may cross talk via Nbp2 (Ohikuni *et al*., 2003). Yet another recent publication discusses the connections of CWI and HOG pathway during zymolyase-induced cell wall stress (Garcia *et al*., 2009). We speculate that Nbp2 may not only be a negative regulator for the HOG pathway, but also for another parallel signalling cascade with cross-talk potential. This other pathway may, when active, suppress HOG specific signalling, and may itself be stronger negatively regulated by Nbp2 than the HOG pathway. Therefore, the signalling may be redirected in *Δnbp2* strains to the other pathway, leading to reduced activation of the HOG pathway. This other pathway could be the FG pathway: Sharing upstream components, the FG MAPK Kss1 was shown to repress Hog1 upon activation of the FG pathway (Yang *et al*., 2009). Nbp2 may negatively regulate Kss1 besides its impact on Hog1. If Nbp2 is deleted,

Hog1 dependent transcription may be lowered, because aberrant activation of Kss1 that in turn represses the HOG pathway.

This idea of competing pathways by regulating each other is further supported by several large scale studies analysing protein-protein interactions (Tong *et al*., 2001; Jorgensen *et al*., 2002; Tong *et al*., 2004), where Nbp2 was found to interact with several proteins also involved in FG, CWI and cytoskeleton organisation (e.g. Bni1, Gas1, Cap2, Sem1). This is an impressive example for the complex composition of signal transduction, and its tightly netted positive and negative feedback that results in one protein although being a negative regulator to be crucial for optimal signal transduction.

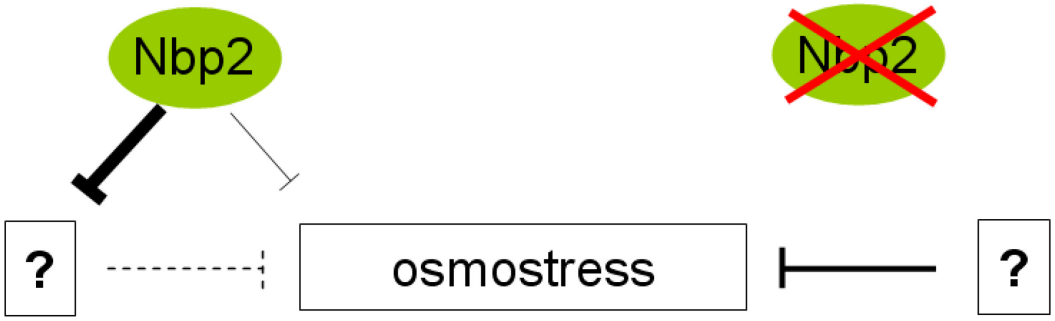


Figure 31: Nbp2 and its regulatory function on MAPK signalling. Upon osmostress application, Nbp2 may repress not only the HOG pathway, but also another repressor of the osmostress signalling cascade. Upon deletion of Nbp2 the second repressor may be fully active and repressing the HOG specific response.

7.4 The SHO1 branch – a model

In summary, putting our data into context with the recent literature of Hog1 pathway signalling (Tatebayashi et al., 2006), we want to propose the following model, shown in figure 32:

During vegetative growth the highly glycosylated proteins Msb2, Hkr1 and Sho1 are in their normal conformation, and loosely associate at the membrane. Furthermore, Pbs2 has high affinity to Sho1. Upon osmotic stress, the glycosylated proteins undergo a conformational change, which activates them. Subsequently, Ste11 becomes activated by phosphorylation of Ste20 that is in turn dependent on Cdc42. Consequently, the on-rates of the interaction of Ste11 and Sho1 become significantly increased. This crucial step is dependent on the action of Ste50, Ste20 and Msb2/Hkr1. Upon activation of Hog1 by Pbs2 the affinity of Pbs2 to Sho1 becomes reduced leading to a collapse of the aggregates. This

step permits the reforming of the complex with a not yet activated Pbs2 molecule. Subsequent reactivation of Pbs2 further amplifies the SHO1 branch signal.

Unfortunately, the exact role of Opy2 and Nbp2 in this process remained elusive. However, the data presented in this thesis allows us to propose some ideas: Opy2 may not be involved in changing affinities of proteins during osmostress, but may rather be required for the correct positioning of signalling components towards each other that interact independently of Opy2. Nbp2 may also have dual function in osmostress signalling: Whereas it may act as a negative regulator of Hog1 after adaptation to osmostress, it may also negatively regulate other MAPK signalling cascades that in turn have the potential to repress HOG signalling.

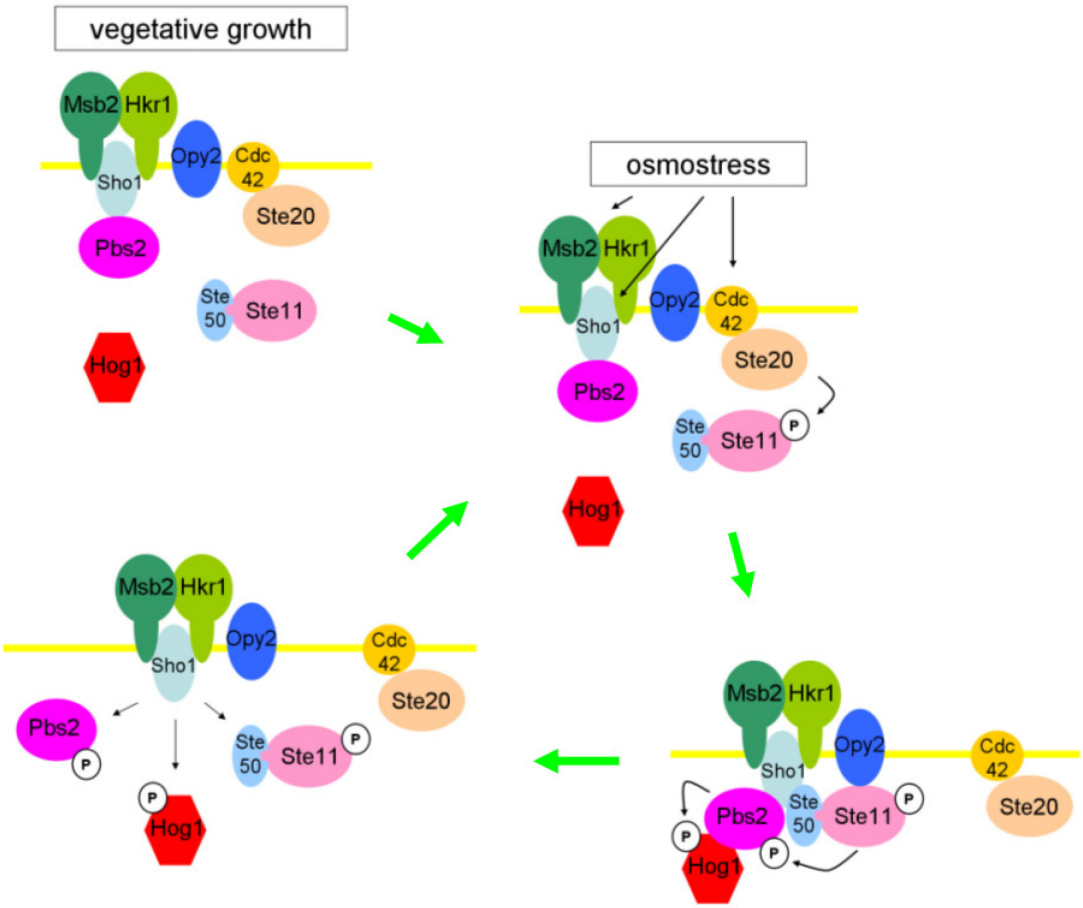


Fig 32: The SHO1 branch – a model. See text for description.

7.5 Outlook

In summary, proteins knit a tight network for correct signal transduction in space and time, where association, dissociation, localisation and activation work together to provide the correct response for a certain stimulus. Signal transduction is not binary events where binding or non-binding of proteins decides of pathway activity, but differences in kinetics, and only if a certain threshold is passed the balance is shifted towards the activation or deactivation of a signalling pathway.

The work presented here clearly shows that the methylation assay is a very useful tool to directly measure the dynamic interactions of proteins. This method is not restricted to studies on PPI in signal transduction: Currently, the assay is successfully applied to study PPIs that are involved in peroxisome biogenesis (C. Brocard, unpublished data). As already mentioned above the procedure can be further refined for certain questions. Ideally, fusion proteins should be expressed from the endogenous locus. However, in most cases this will require enrichment of the H3 epitope to be able to gain detectable signals. In the work presented here we have already shown that we could successfully purify the acceptor-protein via an IP step before applying western analysis. Moreover, initial adaptations of the protocol to be able to use MS as a readout method are already undertaken. As already mentioned above, this will allow differentiation of mono-, di and tri- methylations, thereby allowing the gain additional information about the kinetics of the studied PPIs. Furthermore, different mutant HMTs with different methylation kinetics may further improve this method.

Clearly, this work opened a lot of questions that still need to be answered. One of the most thrilling questions is how Nbp2 acts on the HOG pathway, and also on other MAPK cascades of budding yeast. Further characterisation of the P/A mutant, as well, as a deletion analysis should reveal which regions of Nbp2 are important for interactions with different components of signal transduction cascades.

Also Opy2 has recently become a protein of high interest since it has been found to be necessary for HOG pathway signalling and may also be of importance for other MAPK cascades. It will be interesting to see, if osmostress signalling is aberrantly transduced through a different pathway (e.g. the mating pathway) in the absence of Opy2.

We also strongly believe that the function of Rtn2 is in dear need of being explored further. Rtn2 possibly has a dual role in signal transduction and in the process of osmostress adaptation. It will be interesting to investigate Rtn2's functions in the HOG pathway and in

the FG pathway. Rtn2 may be a component shared by both pathways, or even be a regulator to distinguishing one from the other.

Last, it may be an interesting topic to study signalling specificity taking temporal parameters into account: While the HOG pathway has to be fully activated within minutes, the mating and also the FG pathways have a slower response profile to the respective stimulus. Therefore, it may be that a part of the specificity of signal transduction is the timing of events. Activation peaks at early or late time points may lead to specific induction of the one or the other pathway respectively. Furthermore, by negatively regulating each other during time, only one of the signalling cascades can become dominant over the other. However, induction and repression events cannot be seen separately; all those processes act together in a complex network: First, only molecules in the correct protein context become activated. Second, due to this activation the abundance of the specific stimulated complexes increases. Third, only upon positive feedback of the correct, and suppression of the incorrect signalling pathway, a competent response is developed. Since our methylation assay can potentially track changes in affinity over time, it may prove to be extraordinarily useful for studying the impact of time on signalling specificity.

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