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Analysis of causal connections within the MAPK pathways in yeast

Bachelorarbeit

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List of abbreviations

APS	ammonium persulfate
BSA	bovine serum albumin
ddH_2O	double-distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated protein kinases
fwd	forward
GFP	green fluorescence protein
GTP	guanosine triphosphate
h	hour
LB	lysogeny broth
min	minute
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGK	phosphoglyceratekinase
PVDF	polyvinylidene fluoride
Ras	rat sarcoma
RAF	rapidly accelerated fibrosarcoma
rev	reverse
rho	ras homologue
rpm	rounds per minute
RT	room temperature
sec	second
SD	synthetic dextrose
SDS	sodium dodecyl sulfate
SOC	super optimal broth with catabolite repression
SORB	sorbitol
ssDNA	single stranded DNA
TEA	tris base, EDTA, acetic acid

TBS	tris-buffered saline
TBST	tris-buffered saline with Tween 20
TEMED	tetramethylethylenediamine
YPD	yeast extract peptone dextrose

Zusammenfassung

Die Mitogen-aktivierte Protein Kinase (MAPK) Kaskade stellt eine der am höchsten konservierten Signalmodule in Eukaryoten dar und dient als zentrales Element zur Regulation von Proliferation, Differenzierung, Apoptose und Anpassung an Stress. Über diese MAPK Kaskade werden extrazelluläre Signale in den Zellkern weitergeleitet, in dem dann die Expression vieler signalspezifischer Gene initiiert wird.

In der Hefe *Saccharomyces cerevisiae* sind die Prozesse der Signalweiterleitung im Verpaarungs- und im HOG-Signalweg weitgehend aufgeklärt. Dennoch sind gerade regulatorische Mechanismen in beiden Signalwegen, wie z.B. negative Feedback-loops, oder die Isolation beider Signalwege untereinander größtenteils unerforscht.

Die vorliegende Bachelorarbeit befasst sich mit der Frage, wie die Einleitung einer der beiden Signalwege durch unterschiedliche extrazelluläre Signale zu einer spezifischen Antwort führt, obwohl sich beide Signalwege viele vorgeschaltete Proteine teilen, insbesondere die MAPKKK Ste11. In der Studie von Hecker *et al.* wurde ein negatives Feedback von Fus3 auf Ste11 untersucht. Um sich weiterhin mit diesem regulatorischen Mechanismus zu befassen wurde der Einfluss auf den HOG Signalweg und ein „Crosstalk“ im MAPK Netzwerk untersucht.

Zunächst wurde der Einfluss einer Runterregulierung von Ste11 im Verpaarungs- und im HOG-Signalweg untersucht, indem die Aktivität der jeweiligen terminalen MAPKs Hog1 und Fus3 analysiert wurde. Die Beobachtungen lassen vermuten, dass beide Signalwege durch eine Phosphorylierung auf Ste11-T596 desensibilisiert werden und, dass die beschriebene Feedback-Phosphorylierung auf Ste11-S243 durch Fus3 zu einer kurzzeitigen Aktivierung von Fus3 nach Stimulation mit Pheromonen führt.

Um genauer verstehen zu können, wie Signalspezifizität gewährleistet wird, obwohl sich beide Signalwege viele Proteine teilen, und welcher Schritt einen „Crosstalk“ verhindert, wurden die Aktivitäten der MAP Kinasen Hog1 und Fus3 nach Stimulation mit entweder Pheromonen oder NaCl untersucht. Dabei konnte gezeigt werden, dass osmotischer Stress nicht zu einer Einschaltung des Verpaarungssignalweges führt; jedoch sind die untersuchten Phosphorylierungen auf Ste11 nicht für diese Signalwegisolierung verantwortlich. Des Weiteren wurde festgestellt, dass die Phosphorylierung auf Ste11, zumindest auf T596, eine Aktivierung von Hog1 durch Stimulation mit Pheromonen verhindert.

Um die Ergebnisse in einem komplexeren Zusammenhang sehen zu können, wurde ein dritter Ste11-abhängiger Signalweg, der filamentöse Signalweg (FG), untersucht, indem die

Aktivierung der MAPK Kss1 unter hoher Osmolarität oder nach Stimulation mit Pheromonen detektiert wurde. Diese Analyse ergab, dass der FG Signalweg durch beide Inputs eingeleitet wurde, wenn eine Phosphorylierung von T596 in Ste11 nicht möglich ist.

Abstract

The mitogen-activated protein kinase (MAPK) cascade is one of the most conserved signalling modules in eukaryotic cells and serves as a key element regulating proliferation, differentiation, apoptosis and adaption to external stress. Via the MAPK cascade extracellular signals are transduced to the nucleus initiating the expression of a numerous of response specific genes.

In the yeast *Saccharomyces cerevisiae* MAPK signalling in the pheromone response and the high-osmolarity glycerol (HOG) pathways are in principle well understood. However, several regulatory mechanisms in both MAPK pathways, like negative feedback loops, or isolation from each other still remain unclear.

In this bachelor thesis the question was addressed how activation of the pathways by distinct extracellular signals leads to a pathway specific output despite both pathways share several upstream components, in particular the Ste11 MAPKKK. In the study of Hecker *et al.* a negative feedback from Fus3 on Ste11 was reviewed. To further address this regulatory mechanism the influence on the HOG pathway and crosstalk within the MAPK network was investigated.

At first the influence of Ste11 down-regulation in the HOG and the pheromone response pathway were examined by detecting the activity of the terminated MAPKs Hog1 or Fus3 MAPKs, respectively. These observations suggest that both MAPKs and thus both pathways are desensitized through a phosphorylation of Ste11-T596. In agreement with Hecker *et al.* it was shown that a negative feedback on Ste11-S243 by Fus3 itself is required for a transient Fus3 activation upon pheromone stimulation.

To get a deeper understanding how pathway specificity is ensured despite shared components and at which steps crosstalk between the pathways is prevented Hog1 and Fus3 activity was examined by stimulation with either pheromones or high external osmolarity. It was shown that osmotic stress does not induce mating response but the investigated negative phosphorylation on Ste11 is not required for pathway insulation. However, the regulation via phosphorylation on Ste11, at least on T596, prevents Hog1 activation upon pheromone stimulation.

Furthermore, to see these results in a more complex context, a third Ste11-dependent pathway, filamentous growth pathway (FG), was examined by detecting Kss1 MAPK activity upon high external osmolarity or alpha factor stimulation. Interestingly, the FG pathway is initiated by both inputs when phosphorylation to T596 in Ste11 is disrupted.

1. Introduction

1.1 Signaling via MAP kinase pathways

Adaption to environmental changes is an essential ability for survival of living cells.[1] A highly conserved signaling cascade in eukaryotic cells contains members of the mitogen-activated protein kinase family and transduces detected stimuli from the cell surface to the nucleus. Three protein kinases serve as the key elements of a MAPK pathway, which are activated upstream by (dual-) phosphorylation and (dual-) phosphorylate each other further downstream (Fig.1).[1,2,3]

Transmembrane receptors or cell surface proteins sense extracellular molecules e.g. hormones or growth factors transmitting environmental changes. Initially, the MAP kinase kinase kinase (MAPKKK or, in mammalian cells, RAF) is activated by dual-phosphorylation of either a small GTPase from Ras or Rho family or by a member of p21-activated protein kinase (PAK) family, as called MAP kinase kinase kinase kianse. Upon activation the MAPKKK phosphorylates conserved serine and threonine residues in the T-X-Y motif in the activation loop of the MAP kinase kinase (MAPKK or MEK), which in turn phosphorylates a threonine and a tyrosine located in the activation loop of the MAP kinase (MAPK or ERK).[1,2,4] A transient accumulation of activated MAPK in the nucleus induces the activation of several substrates, regulating gene expression, cellular morphology, cell cycle progression and metabolism e.g. transcription factors or MAPK-activated protein kinases.[1,4] Thus, via MAPK networks cells regulate proliferation, apoptosis, differentiation and are able to respond to diverse extracellular stimuli.[5]

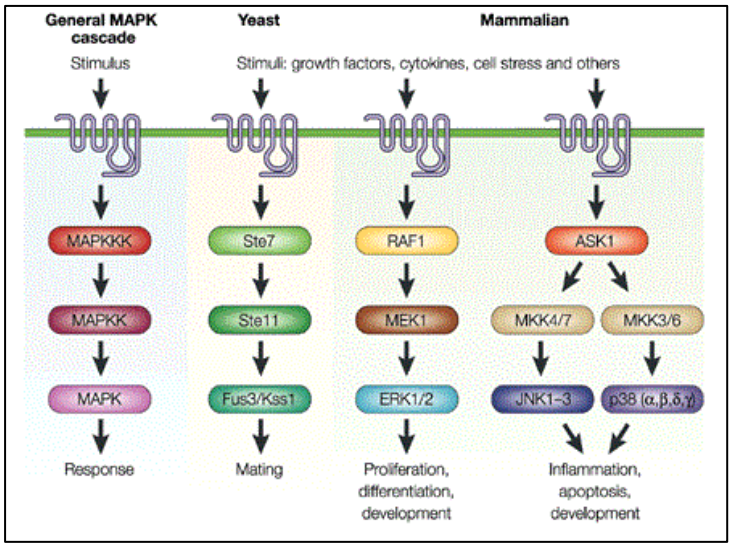

Figure 1: Architecture of a typical MAPK signal module in yeast and mammalian cells.[6]

1.2 MAPK pathways in yeast

In the budding yeast *Saccharomyces cerevisiae* five signaling pathways based on MAPK cascades are known: the cell-wall integrity pathway, the spore wall assembly pathway, the filamentous growth (FG) pathway, the pheromone response pathway and the high osmolarity glycerol (HOG) pathway.[2,5,7]

Interestingly, three of these pathways (FG, HOG and pheromone response) share the same upstream proteins and signal via the same kinases. Ste11 serves as a common MAPKKK, which is activated by upstream Ste20, Ste50 and Cdc42.[1,5,8] Further, the FG and the pheromone response pathway use a common MAPKK: Ste7.[1,9]

1.3 High osmolarity glycerol pathway

1.3.1 Signalling in the HOG pathway

Cells respond to an increased extracellular osmolarity by activating the high osmolarity glycerol MAPK pathway containing the effector MAPK Hog1.[1,8,10] A higher external than internal level of dissolved solute reduces cellular turgor pressure. Activation of the HOG pathway induces cell volume growth by increased cellular biosynthesis of glycerol that strongly dissolves water.[10,11]

The transmembrane complexes Sho1 and Sln1 serve as receptors activating either one of the two branches of the HOG pathway.[7,12] Additionally a third osmosensing branch was discovered that may signal similar to the SHO1 branch (Fig. 2).[13,14]

The osmosensor Sln1 is under iso-conditions in a permanently active state catalysing its autophosphorylation.[1,8] This phosphate is further transferred to an intermediate phospho-carrier protein, called Ypd1, and subsequently to the response regulator protein Ssk1. Phosphorylation of Ssk1 prevents its interaction with the MAPKKKs Ssk2 and Ssk22, which are required for activation of the MAPKK Pbs2. High external osmolarity causes an inhibition of Ssk1 that binds and consequently activates the autophosphorylation of Ssk2 and Ssk22 activating Pbs2.[7,10,15]

In contrast to the SLN1 branch the mechanism of responding to osmotic stress via Sho1 is still not completely understood.[1,7] One possibility to activate Pbs2 in this branch is via a direct interaction between Ste11 and a C-terminal region of the osmosensor Sho1.[1] Other reports indicate that the adaptor protein Ste50 is essential for signal transduction in the SHO1 branch. Sho1 consists of four transmembrane segments near its N-terminus whereas its C-terminal region is a cytosolic SH3 domain with high affinity to Pbs2. The interaction between Sho1

SH3 domain and the N-terminal proline-rich motif in Pbs2 causes the phosphorylation of Pbs2 by Ste11. The presence of Ste50 is required for Pbs2 activation as it directs Ste11 and Pbs2.[1,3] Another variation of activating Pbs2 through the Sho1-Ste11/Ste50 branch is via Opy2 and Ste50. Opy2 is another transmembrane protein and may function as a membrane anchor recruiting Ste50 to the membrane that in turn associates with Ste11. Ste20, that is also anchored at the plasma membrane by Cdc42 GTPase, phosphorylates Ste11 which in turn activates Pbs2.[1,16]

Recent studies identified a third osmosensor containing the highly glycosylated osmosensor Msb2. Probably Msb2 activates the components downstream of Sho1 which confirms the existence of only two mechanisms eventually activating Pbs2.[13,14]

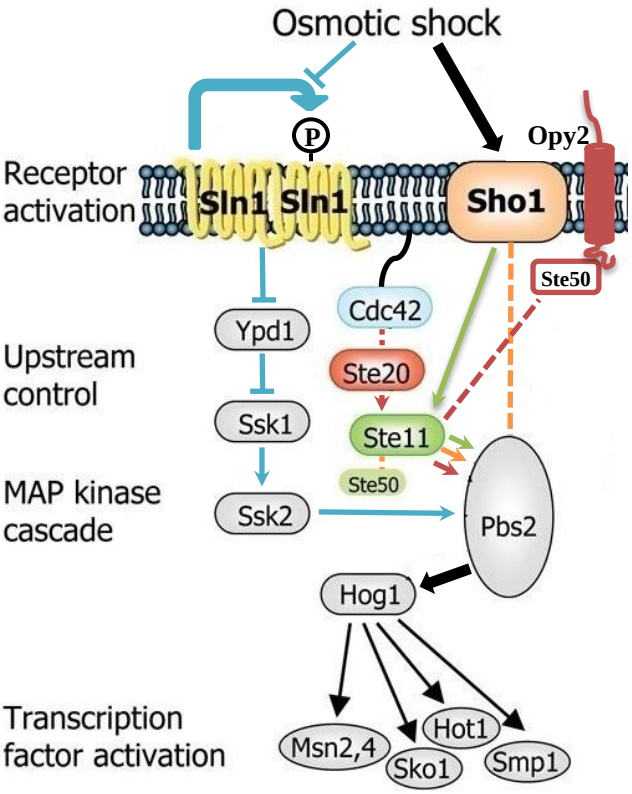

Figure 2: Scheme of the HOG pathway in yeast induced by high external osmolarity. SLN1 branch is shown with blue arrows. Different ways of signal transduction in the SHO1 branch are shown with green, red and orange arrows. All cases end up in Pbs2 activation, which in turn activates Hog1 that accumulates in the nucleus. Broken lines show complex formation. T-bar arrows indicate inhibition. Adapted from Klipp *et al.*[17]

In all branches dual-phosphorylation by Psb2 activates Hog1 MAPK and induces its rapid translocation to the nucleus.[1,7,10,11] Further transcription factors e.g. Hot1, Smp1 and RNA polymerase II are phosphorylated and the expression of several genes for osmoadaption is activated.[18] Hot1 regulates the expression of GPD1 and GPP2 genes that encode enzymes (glyceraldehyde-3-phosphate dehydrogenase and glyceraldehyde-3-phosphatase) catalysing the converting of glyceraldehyde-3-phosphate to the osmolyte glycerol.[10,11] Additionally the

expression of STL1 gene is induced encoding the glycerol active transporter Stl1. This glycerol/H^+-symporter is responsible for glycerol import, which represents a second response of cellular glycerol accumulation to an osmotic shock.[19]

In order to recover to the normal cell growth after responding to hyperosmotic stress several inhibitory mechanisms are known demonstrating the high dynamics of the HOG network (Fig.3).[8,10]

The protein tyrosine phosphatase Ptp2, which is mainly located in the nucleus, is able to remove the tyrosine bound phosphate of Hog1. Further reports discovered a negative feedback mechanism preventing an excessive activity of Hog1 upon high osmolarity since active Hog1 causes an increase of Ptp2 activity.[2,8,11]

Furthermore protein phosphatases type 2C (PP2C) dephosphorylate threonine residues. Ptc1 may form a complex containing Nbp2 (small adaptor protein), Pbs2 and Hog1 resulting in inactivation of Hog1. In that case the SH3 domain of Nbp2 is bound to a proline-rich motif of Pbs2, which is distinct to the binding site of the osmosensor Sho1.[20,21]

A recent study of Yamamoto *et al*. indicates the existence of a negative feedback from active Hog1 to SHO1 branch. In that case Ste50 is phosphorylated and thereby its affinity to Opy2 is reduced.[22] Previously O'Rourke *et al.* reported on a feedback phosphorylation to Sho1 by pHog1.[23] Further studies (Hao *et al*.) suggest that the rapid feedback phosphorylation from active Hog1 to both Ste50 and Sho1 limits cross talk to FG pathway.[24]

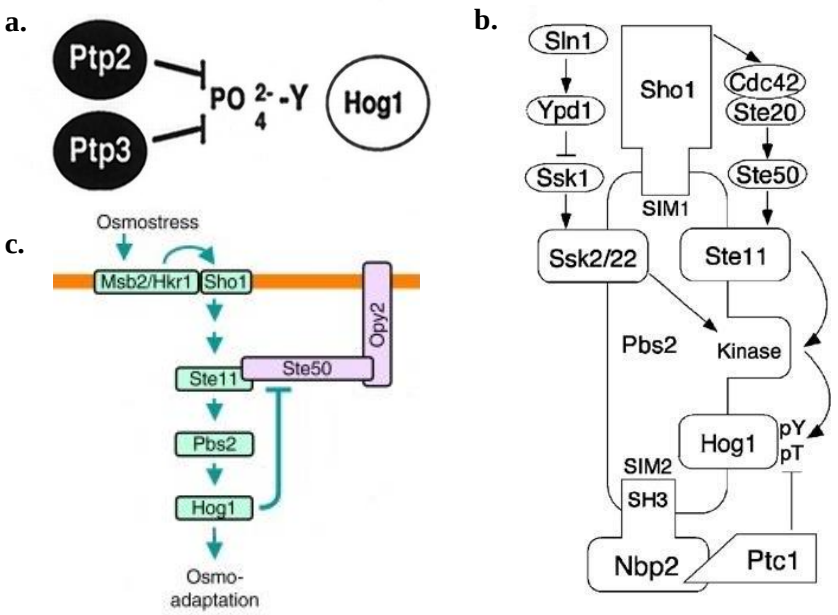

Figure 3: Scheme of Hog1 down-regulation upon high osmolarity. **a.** by PTPs or PP2Cs via dephosphorylation of its tyrosine residue. **b.** by Ptc1 via a complex formation containing Nbp2 and Pbs2. **c.** via a negative feedback from active Hog1 by phosphorylation of Ste50 reducing its affinity to Opy2 and thus preventing crosstalk to FG pathway.[25,21,22]

1.3.2 Crosstalk between the HOG and other MAPK pathways

Another study claims that HOG pathway and pheromone response pathway are insulated from each other because both use a specific scaffold/docking protein (Pbs2 and Ste5), which might be a general strategy for cross talk prevention (Fig.4).[5,26,27] Further, no significant output from FG pathway upon high osmolarity is detected although the pathways share many upstream components. Indeed Kss1 is activated by Ste7 during osmotic stress, but Hog1 is able to inhibit DNA-binding of FG specific transcription factor Tec1.[5,28]

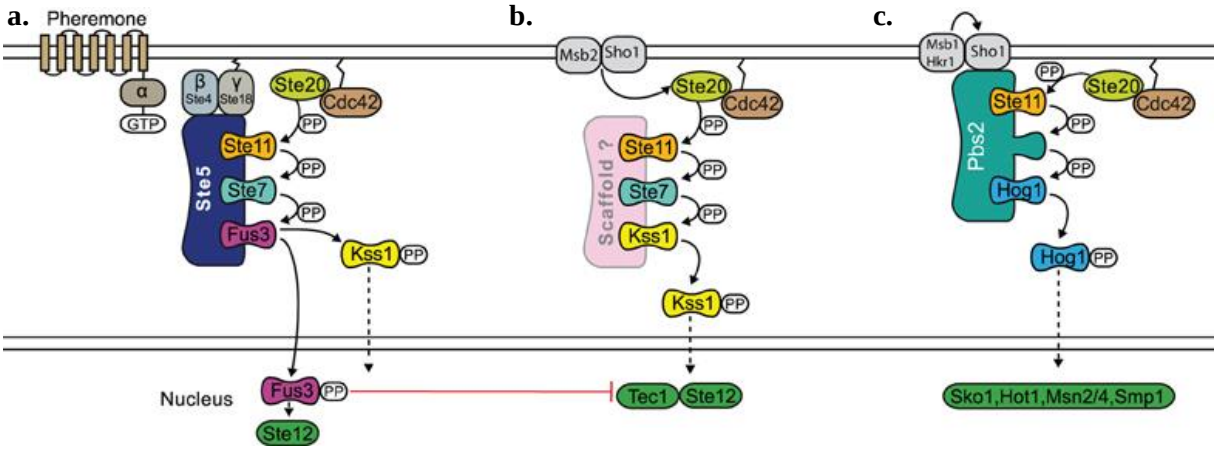

Figure 4: Scheme of crosstalk inhibition via specific scaffold proteins in the **a.** pheromone response pathway. **b.** the FG pathway and **c.** the HOG pathway in yeast, although they share the Ste11 MAPKKK. However other mechanisms are required to ensure pathway specificity.[29]

1.4 Pheromone response pathway

1.4.1 Signalling in the pheromone response pathway

In budding yeast mating of haploid cells is regulated via a MAPK cascade: the pheromone response pathway (Fig.5). *S. cerevisiae* exists either as haploid **a** cells or haploid **α** cells. Both cell types sense each other by the release of peptide pheromones (**a** cells release a-factor; **α** cells release α-factor). Binding of α-factor to Ste2 (in **a** cells) or a-factor to Ste3 (in **α** cells) receptor induces mating. Eventually the Fus3 MAPK is activated, which causes expression of mating-specific genes, initiates cell cycle arrest and in general prepares the cell for undergoing cell-cell fusion and thus forming a diploid cell.[1,30,31]

Upon stimulation of either Ste2 or Ste3 each monomeric receptor serves as guanine nucleotide exchange factor (GEF) resulting in the exchange of GDP to GTP in the coupled heterotrimeric G-protein (Gαβγ complex), which is then active. This regulates the dissociation of Ste4-Ste18-subunits (Gβγ) from GTP-Gpa1 (Gα). The Gα-subunit remains bound to the receptor and the activated Gβγ complex migrates along the membrane and now is able to recruit the

Ste5/Ste11 complex, Far1 and the MAPKKKK Ste20. Far1 binds both the Gβγ complex and recruits Cdc24 to the membrane, which serves as GEF of Cdc42. Active GTP-Cdc42, which is anchored to the membrane, then binds and activates a member of the PAK family, Ste20. Subsequently Ste11 is not only activated via phosphorylation by Ste20 but probably also requires binding of Ste50 to Ste11 which further induces the MAPK module (Ste11 → Ste7 →Fus3).[1,31,32] Ste5 scaffold protein binds Ste4 and locally tethers all three MAPKs and brings them near to the plasma membrane.[33,34,35]

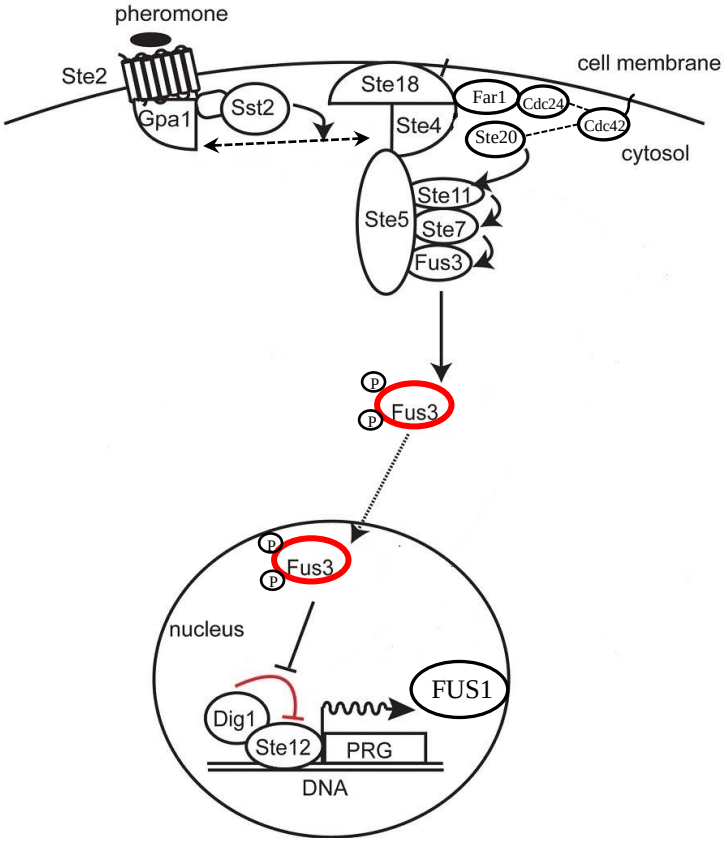

Figure 5: Scheme of pheromone induced MAPK pathway in yeast. Central MAPK Fus3 is marked in red. Black arrows indicate activation; broken lines indicate complex formation; broken double-headed arrow shows complex formation or dissociation, in case of activation. Adapted from Yu *et al.*[36]

As a result Fus3 translocates to the nucleus where it inhibits Dig1 and Dig2, which are negative regulators of the transcription factor Ste12. Further, proteins which are required for shmoo tip formation, e.g. Fus1, are expressed and accumulated on the membrane.[31,37]

1.4.2 Regulatory mechanisms within the pheromone response pathway

As the HOG pathway the pheromone response pathway can be inactivated by protein phosphatases. Ptp3 which is mainly cytoplasmic removes a phosphate of Fus3.[2,8]

Further the mating pathway is regulated via several negative feedbacks (Fig.6). In all these mechanisms Fus3 MAPK controls upstream components.[31,36]

One mechanism involves the Regulator of G-protein Signaling (RGS)-protein Sst2, which probably promotes Ste5 membrane recruitment. Fus3 negatively regulates Sst2-Ste5 interaction ensuring the dose-response alignment in the pheromone response pathway.[36]

Furthermore the activity of the dual-specificity phosphatase (DSP) Msg5 is induced by active Fus3 and causes the removal of both phosphates of Fus3. It was shown that Msg5 is mainly responsible for supporting a low basal phosphorylation of Fus3.[2,8,31]

Further studies claim that the ultrasensitive switch-like activation of the pathway is a result of a competition for phosphorylation sites on Ste5 between Fus3 and Ptc1. Dephosphorylation of Ste5 by Ptc1 reduces affinity of Fus3-Ste5 binding.[5,33]

Recent works report on another negative feedback induced by Fus3 reducing activity of the homologous Kss1 MAPK (FG pathway) preventing a leakage of the pathway. This signal specificity is induced via a feedback from Fus3 to Ste7 MAPKK, which is shared by both pathways.[38,39]

A so far unknown negative feedback mediated by Fus3 was observed by using Fluorescence Correlation Spectroscopy (FCS) detecting interactions of kinases with their substrates and the scaffold protein Ste5. The study showed a feedback phosphorylation of the upstream Ste11 MAPKKK by Fus3 MAPK. Further analysis indicated that this negative control is responsible for the all-or-none behaviour of the mating response and additionally limits cytosolic localization of Fus3 to form a single shmoo.[40]

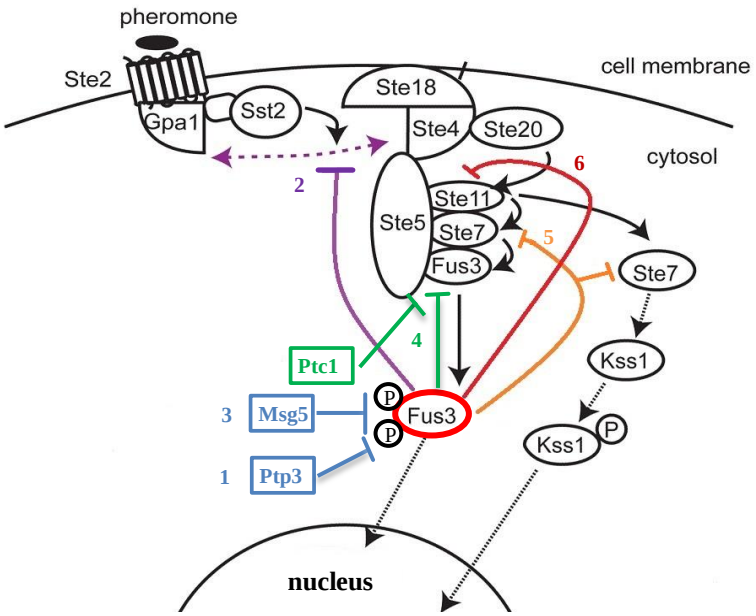

Figure 6: Scheme of different ways to inactivate the mating pathway in yeast described more detailed above. T-bar arrows indicate feedback mechanism; black arrows indicate signal transduction; broken lines indicate complex formation or translocation. **1.** removal of Fus3 Y-phosphate through Ptp3. **2.** negative regulation of Sst2-Ste5 interaction by pFus3. **3.** removal of both phosphates of Fus3 by Msg5. **4.** active Fus3 down-regulates its activity by inhibiting its interaction with Ste5 and competing with Ptc1 **5.** crosstalk prevention through FG pathways by negatively regulation of Ste7 by pFus3. **6.** Phosphorylation of S243 residue of Ste11 which probably leads to a down-regulation of several MAPK pathways by active Fus3. Adapted from Yu *et al.*[36]

1.4.3 Crosstalk between the pheromone response and the FG pathway

Several studies report on a leakage in pheromone response pathway to the FG pathway, which is induced in the absence of nutrients leading to elongated cells. This observation is based on the sequence analogy of Fus3 and Kss1 MAPKs (about 55 % amino acid identity[41]) and on the shared upstream components.[5]

Decision making in the yeast pheromone pathway is characterized by a switch like Fus3 phosphorylation requiring Ste5, Ste7 and Fus3.[5,33] In contrast, the FG pathway shows a more linear increase of the response depending on the concentration and does not require a scaffold protein.[5,9] These facts result in activation of Kss1 by Ste7 at low pheromone concentrations. But pheromone induced Kss1 activation is weaker and more transient than Fus3 activation.[5] In contrast, the absence of nutrients does not activate pheromone signaling as Ste5 is not involved in FG pathway. However pheromone response pathway prevents FG gene expression since activated Fus3 phosphorylates the FG specific transcription factor Tec1 which in turn is degraded and gene activation is blocked.[29,38]

1.5 Central role of the Ste11 MAPKKK

1.5.1 Involvement of Ste11 in three MAPK pathways in yeast

Ste11 is part of a MAPK cascade in three signalling pathways in *S. cerevisiae*: the FG pathway, the pheromone response pathway and the HOG pathway (Fig.7).[1,5,7]

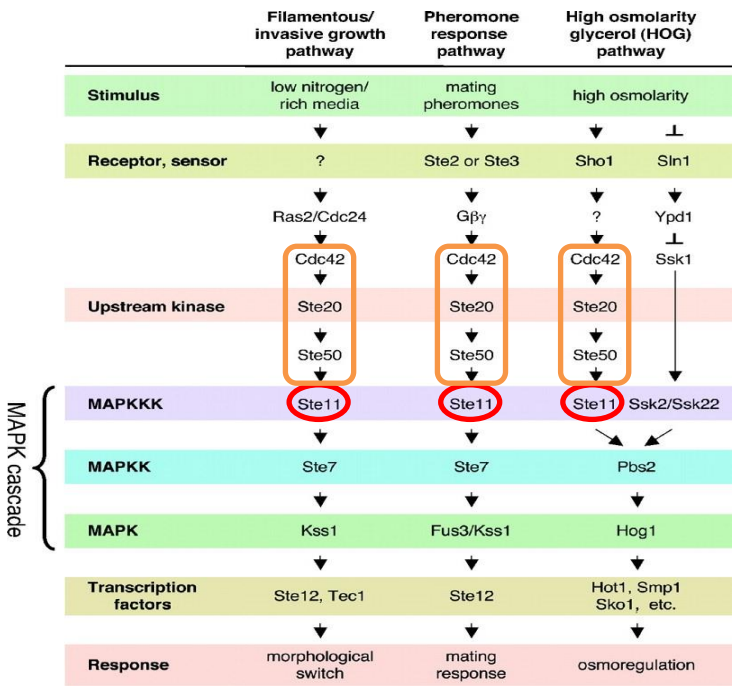

Figure 7: Scheme of FG, pheromone response and HOG MAPK pathway in yeast illustrating the involvement of numerous upstream proteins and in particular Ste11 in three pathways; the central element Ste11 is highlighted in red, upstream architecture is marked in orange. Adapted from Saito *et al.*[8]

Interestingly, all pathways respond to different stimuli and lead to activation of completely independent molecular and cellular processes. The upstream architecture of Ste11 activation consists of the same proteins: Cdc42, Ste20 and Ste50.[1,5,8]

But how is signal specificity in these pathways ensured in the cell? A leakage might be prevented by several negative feedback mechanisms, which are up to now little understood. Some were mentioned above but so far no feedback mechanism on Ste11 is known.

1.5.2 Structure of Ste11 - catalytic and regulatory domains

The crystal-structure of Ste11 is still not solved. Numerous studies report that Ste11 consists of an overall C-terminal kinase domain and three regulatory domains located near the N-terminus (Fig.8).[3,31]

The regulatory domain contains a conserved sterile alpha motif (SAM) domain that heterotypically associates with the N-terminal SAM domain of the adaptor protein Ste50. In addition the SAM domain in Ste11 is followed by a catalytic-binding domain (CBD) and phosphorylation sites that are controlled by Ste20.[31,42]

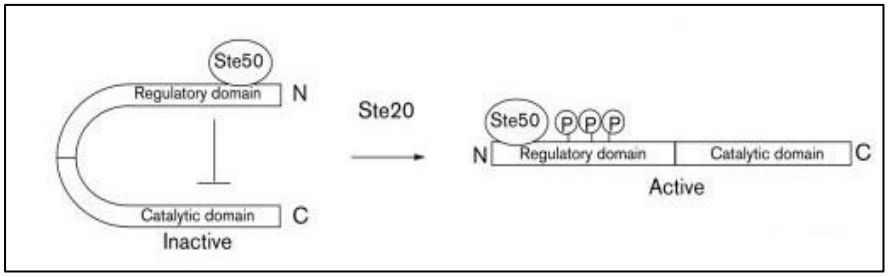

Figure 8: Scheme of Ste11 auto-inhibition and activation by binding of Ste50 and phosphorylation on S302 and/or S306 and T307 by Ste20. Adapted from v. Drogen *et al.*[3]

A mutational analysis was done showing that inactive Ste11 inhibits itself (Fig. 4). In this model the CBD of inactive Ste11 inhibits its catalytic domain by binding. Responding to an extracellular stimulus Ste20 phosphorylates either two or three amino acids near the N-terminus of Ste11, which causes the dissociation of the inactive conformation.[3] It has been reported that binding of Ste50 to the SAM domain of Ste11 is also required for abolishment of self-inhibition.[3,7] In summary binding of Ste50 to Ste11 and phosphorylation by Ste20 lead to a transduction of the mating signal.

1.5.3 Ste11 mutants

In order to analyse the role of Ste11 in the negative feedback mechanism from Fus3 to Ste11 two mutant strains were used in Hecker *et al*.. Interruption of this negative feedback is caused

by the replacement of S243 by a non-phosphorylatable alanine. In Ste11 the S243 is located in the binding domain to Ste5. A second mutant on T596, to a non-phosphorylatable isoleucine, leads to a constitutive active Ste11. Currently, it is not known which component phosphorylates T596 in Ste11 to negatively regulate signalling. This site T596 is positioned in the kinase domain of Ste11.

Thus, the following mutants were designed and compared with Wt: Ste11(S243A), Ste11(T596I) and a mutant containing both Ste11(S243A/T596I).[40]

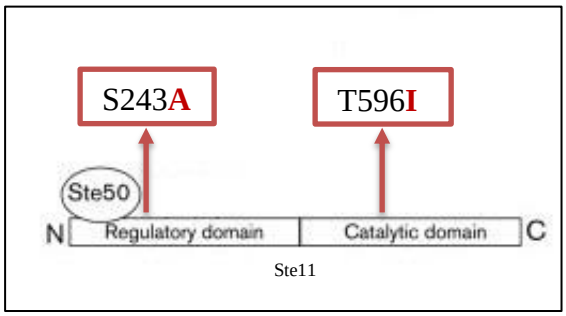

Figure 9: Positions of the two replaced amino acids in Ste11 marked in red in order to investigate a negative feedback phosphorylation on Ste11, which is a common MAPKKK in several pathways in yeast. Adapted from v. Drogen *et al.*[3]

With these mutants it was observed that non-phosphorylatable Ste11 leads to an increased FUS1 gene expression and changes in cell morphology. Interestingly, already unstimulated Ste11(S243A/T596I) double mutant cells were unsymmetrically elongated and showed a higher basal level of Fus3 phosphorylation. Ste11(S243A) cells responded more sensitive to low pheromone concentrations and also exhibited shmoo formation at lower dose of alpha factor than Wt. Unexpectedly the Ste11(T596I) strain showed no noticeable changes in cell morphology.[40]

1.6 Aim of the project

The aim of this work is to quantify the influence of disturbing a negative feedback phosphorylation on the Ste11 MAPKKK in both the high-osmolarity glycerol and the pheromone response pathway in yeast. Additionally, a potential leakage between both pathways via the shared kinase Ste11 should be investigated, which may be prevented through the described feedback phosphorylation. In addition to the analysis of the activity of the corresponding MAPKs Hog1 and Fus3 the influence on gene level using fluorescent-tagged proteins should confirm previous findings.

The activity of either Fus3 or Hog1 and thus the signal transduction of the pathway is represented by phosphorylation of two residues in its activation loop. Time-course experiments of the MAPK phosphorylation status in several mutant strains should show the influence of the disturbed negative feedback on Ste11 though the activity of either Hog1 or Fus3 upon stimulation. Furthermore the insulation of several MAPK pathways in yeast depending on that negative feedback should be analysed. This could help to understand the dynamic of a MAPK system in yeast and can draw conclusions to mammalian MAPK signalling.

In the second part of this work the influence of that negative feedback phosphorylations on gene level in terms of a cross talk between pheromone response and HOG pathway should be analysed. In general activation of a MAPK, such as Hog1 or Fus3, causes its translocation to the nucleus where it activates gene expression depending on the stimulus. Here expression of GFP-tagged Fus1 protein, which is normally induced by mating signal, should be measured upon high external osmolarity concluding a crosstalk between these pathways.

2. Materials and Methods

2.1 Cell biological methods

2.1.1 Yeast growing

25 mL YPD medium were inoculated with the relevant yeast strain and incubated overnight at 30 °C while shacking. In order to quantify the cell growth the OD at 600 nm was determined by taking 100 µL culture and 900 µL YPD. The culture was diluted to an OD_{600} of 0.5. After incubation (about 1.5-2 h) at 30 °C to an OD_{600} 1-1.2 cells were stimulated.

YPD medium

20.0 g bacto peptone
10.0 g yeast extract
20.0 g Glucose
add ddH_2O to 1 L

2.1.2 Yeast stimulation

At first 2 mL cell culture were transferred to 2 mL Eppendorf tubes and stimulated with either 0.2 M NaCl (80 µL of a 5 M NaCl stock) or 100 nM pheromone (2 µL of a 0.1 mM pheromone stock). The samples were shacked at 850 rpm and 30 °C. In order to quantify the protein expression after a stimulus as a function of time the cells were shock frozen with liquid nitrogen at 0´, 5´, 10´and 30´. After thawing the pellet a cells extraction was performed.

2.1.3 Yeast extraction

The pellet was homogenized in 400 µL PE-Buffer and transferred into 1.5 mL Eppendorf tubes. After 3 min of centrifugation at 13,200 g and 4 °C the pellet was dissolved in 40 µL PE-buffer. In order to lyse the cells glass beads were added to the solved pellet and the suspension was alternately incubated on ice (1 min) and vortexed (1 min) for 25-30 min. After that the samples were centrifuged at 13,200 g and 4 °C for 20 min. The supernatant was centrifuged again for 10 min at 13,200 g and 4 °C and the concentration of the protein was determined via Bradford assay.

extraction buffer

20 mL	tris-HCl 1 M stock (pH 7.9)
8.0 mL	NaCl 5 M stock
40 mL	glycerol
4.0 mL	EDTA 250 mM stock
10.2 µL	β-mercaptoethanol
add ddH_2O to 200 mL	

For preparation of yeast protein extraction buffer (PE-buffer) 100 µL Phosphatase Inhibitor Cocktail 2 and 3 and 400 µL Protease Inhibitor Cocktail EDTA free were added per 10 mL extraction buffer.

2.2 Biochemical methods

2.2.1 Bradford assay

The Bradford assay is a method for measuring the concentration of proteins. Thereby proteins were stained and photometrically detected by visible light. The Bradford reagent contains Coomassie Brilliant Blue G-250 that binds through positively charged regions of proteins. This reaction causes a blue coloration that can be detected by 595 nm.

For determination of the concentration of proteins a standard curve was measured. For this 1 µL of a BSA solution (0.0001, 0.0005, 0.0010, 0.0025, 0.0050, 0.0100 ng/mL) were prepared with 499 µL H_2O and 500 µL Bradford reagent. This complies a dilution factor of 1000. After stirring the samples with a pipette the absorption at 595 nm was detected by using a spectrophotometer. The samples of unknown protein concentration were prepared the same way as the standards. For the blank 500 µL of the Bradford reagent diluted in 500 µL H_2O were measured.

In this work each sample was prepared twice and measured.

2.2.2 SDS gel electrophoresis

To separate proteins based on their molecular weight a SDS PAGE (SDS polyacrylamide gel electrophoresis) was done. SDS is binding on hydrophobic regions of folded proteins by destroying their tertiary structure. Moreover all SDS bonded proteins become uniformly negative charged because 1.4 g SDS is binding per gram protein. Besides β-mercaptoethanol reduces disulfide-bridges that additionally destabilises the tertiary structure of proteins. The samples were heated up to 95 °C for about 5 min which also causes a denaturation of the proteins.

Applying voltage (120 V for 20 min than increased voltage of 140 V for 30-40 min) effects a separation of the proteins according to their molecular weight. At first all proteins assemble in the stacking gel in order to get separated by size in the separating gel. Small proteins migrate faster through the gelpores than larger ones. Comparing the bands of the samples with the bands of a protein standard gives information about their molecular weight.

5x sample buffer

0.6 mL	tris-HCl 1 M stock (pH 6.8)
5.0 mL	50% glycerol
2.0 mL	10% SDS
0.5 mL	β-mercapoethanol
1.0 mL	1% bromophenol blue
0.9 mL	ddH_2O

10x running buffer

30 g	tris base
144 g	glycine
10 g	SDS
add ddH_2O to 1 L	

separating gel buffer (1M Tris-HCl, pH 8.8)

30.3 g	tris base
150 mL	ddH_2O
adjust to pH 8.8 with HCl and add ddH_2O to 250 mL	

stacking gel buffer (0,5 M Tris-HCl, pH 6.8)

11.4 g	tris base
150 mL	ddH_2O
adjust to pH 6.8 with HCl and add ddH_2O to 250 mL	

Table 1: Components of the used acrylamide gels (descriptions are for four 1.5 mm gels).

Component	**Separating gel (12 %)**	**Stacking gel (4%)**
30% acrylamide	12.0 mL	1.95 mL
tris-HCl gel buffer	7.5 mL (pH 8.8)	3.75 mL (pH 6.8)
10% SDS	280 µL	150 µL
ddH_2O	10.5 mL	8.4 mL
TEMED	40.0 µL	60.0 µL
10% APS	200 µL	180 µL

2.2.3 Western Blot

To identify specifically proteins on the SDS gel Western Blots were used. It is an immunological method based on the high specificity of antibody-antigen interactions. Proteins initially became immobilized on a PVDF membrane that previously was activated

with methanol. The SDS gel was deposited between a Whatman paper and the membrane. On the other side of the membrane a Whatman paper was placed. Using the Western Blot equipment and applying voltage (100 V) for nearly 1 h causes an immobilization of the separated proteins on the membrane.

In order to prevent unspecific binding of the used antibodies first a blocking step (1 h) with blocking buffer was performed. Afterwards the membrane was shacked overnight in a solution containing primary antibody that specifically binds to the protein of interest.

After the treatment with primary antibody the membrane was washed 3 times with TBST for 5 min. To visualize the favoured protein the membrane was treated with secondary fluorophor associated antibody (1:5000) for nearly 1 h. The treatment with secondary antibodies necessitates a 10 min washing step with TBST (3 times each). For analysis of the Blot the Odyssey Imaging System was used.

In this work for quantification of expression level of several proteins PGK is used as an internal standard. The molecular weight of each protein corresponds to the data on the information sheet of each antibody.

25x transfer buffer

18.2 g tris base
90.0 g Glycine
add ddH_2O to 500 mL

1x transfer buffer

40 mL 25x transfer buffer
200 mL methanol
add ddH_2O to 1 L

10x TBS (pH 7.6)

12.11 g tris base
87.66 g NaCl
adjust to pH 7.6 with diluted acetic acid
and add ddH_2O to 1 L

TBST

1 L 1x TBS
1.0 mL tween 20

2.2.4 Strip Western Blots

In order to analyse another protein on the membrane to compare both under exactly the same conditions the blot can be stripped. For that reason the stripping buffer removes antibodies even though all proteins stay immobilized on the membrane. Thus, another protein can be visualized with specific antibody.

Hence the membrane was shacked for 30 min in a solution containing stripping buffer (1:4) and afterwards it is washed 3 times with TBST (each 10 min). After a blocking step with blocking buffer (30 min) the membrane can be incubated with primary antibody.

2.2.5 Agarose gel electrophoresis

For the analysis of DNA fragments e.g. proving the success of a PCR a gel electrophoresis with an agarose gel was performed. In this work a 1% agarose gel was used, which consisted of 1.0 g agarose solved in 100 mL TAE buffer. In order to detect the DNA fragments 5.0 µL RedSafe per 100 mL TAE buffer were added. Applying voltage (120 V) for nearly 30 min causes a migration of the fragments because of their negatively charged phosphate backbone. Comparing the bands of the 2-Log Ladder with the bands of the samples gives information about the size of several linear DNA fragments.

50x TAE buffer

30.0 g	tris base
57.1 g	acetic acid
100 mL	EDTA 0.5 M stock (pH 8.0)
add ddH_2O to 1L	

10x sample buffer

5.0 mL	100% glycerol
2.0 mL	0.5% orange G
1.0 mL	EDTA 0.5 M stock
1.0 mL	ddH_2O

2.3 Cloning

2.3.1 Transformation of plasmid DNA in chemical competent *E. coli* cells

In order to increase the amount of plasmid DNA e.g. for yeast transformation competent *E. coli* cells were transformed with the corresponding plasmid.

Hence 100 µL chemical competent bacterial cells were placed in 1.5 mL tubes and 3.5 µL of a 2.25 M DTT stock were added and mixed gently. Then 1 µL of the relevant plasmid were added and mixed again gently. After a 30 min incubation on ice the cells were heat shocked for 30 sec at 42 °C in water bath which caused the absorption of the plasmid. The tube was placed immediately on ice for 2 min. Afterwards 400 µL SOC medium were added and the tube was incubated for 1 h at 37 °C and 200 rpm. Then 20 µL bacterial cells were plated on LB medium plates containing 100 µg/mL ampicillin for selection. Additionally 20 µL of an 10-fold dilution were plated on another plate. Both plates were incubated at 37 °C overnight. Next day 5 mL LB medium containing ampicillin (100 µg/mL) were inoculated with a single colony and incubated again overnight while shacking. For Mini preparation of plasmid DNA 3 mL bacterial culture were used.

SOC medium

20.0 g	bacto-tryptone
5.0 g	yeast extract
0.5 g	NaCl
0.186 g	KCl

add ddH_2O to 980 mL (sterilize)

add 20 mL sterile filtered 1 M glucose

LB medium (pH 7.5)

10.0 g	bacto-tryptone
5.0 g	yeast extract
10.0 g	NaCl

add ddH_2O to 1 L

2.3.2 Mini preparation of plasmid DNA

In order to isolate the plasmid DNA the Roti®-Prep Plasmid MINI kit was used. At first 3 mL bacterial culture were centrifuged at 13,200 g and RT for 20 sec. The pellet was resuspended in 250 µL resuspension buffer (containing RNase A) and additionally 250 µL lysis buffer were added. The solutions were mixed by inverting the tube several times. After addition of 350 µL neutralization buffer and inverting again the tube was centrifuged at 15,000 g and RT for 10 min. Du the meantime columns were activated with 100 µL column activation buffer and a centrifugation at 10,000 g for 30 sec. Activated spin columns were placed in 2 mL collection tubes and supernatant of the samples was added in the centre of the columns. By centrifugation at 10,000 g and RT for 30 sec the lysed plasmid DNA is immobilized on the matrix. In the following the columns were washed twice. Once with 500 µL washing buffer and a 1 min centrifugation at 10,000 g and RT and a second time with 750 µL and centrifugation for 2 min. Furthermore the empty columns were centrifuged again at 10,000 g for 1 min, which removes residual ethanol. The plasmid DNA is eluted by incubation with 35 µL elution buffer for 1 min and add-on centrifugation at 10,000 g and 1 min. In order to determine the DNA concentration a NanoDrop spectrophotometer was used.

2.3.3 Making yeast cells competent

For transformation of yeast initially the cells have to be made competent. Hence the cells achieve the ability to absorb DNA.

At first 25 mL YPD was inoculated with yeast a strain of interest and incubated at 30°C overnight while shacking. Next day the culture was diluted to an OD_{600} of 0.5 and incubated again for nearly 2 h to an OD_{600} of 1-1.5. After centrifugation at 500 rpm and RT for 5-10 min the pellet was resuspended in 5 mL sterile H_2O and centrifuged again. The pellet was dissolved in 5 mL SORB medium and centrifuged for the third time. Afterwards cells were resuspended in 180 µL SORB medium. In addition 35 µL ssDNA were added, which

previously was heated up to 95°C for 5 min. The competent cells were stored as 100 µL aliquots at -80°C.

SORB medium (sterile filter)

100 mM LiOAc
10 mM tris/HCl (pH 8.0)
1.0 M Sorbitol
1.0 mM EDTA/NaOH (pH 8.0)
adjust to pH 8.0 with diluted acetic acid

2.3.4 Epitope gene tagging based on PCR

For the analysis of distinct proteins in vivo it is useful to tag desired proteins with fluorophores. The genetical modification can be done by homologous recombination witch works well in yeast cells. In this work proteins were C-terminal tagged with a PCR based method.

Hence a part of a cassette plasmid including the relevant tag e.g. the sequence of triple mCherry and a selection marker is amplified (in this first pMK20 was used, than pYM34). The used primers consist of the sequence located directly forward or after the inserting sequence and an overhanging gene. Consequently the sequence (40-60 bases) matches either the last C-terminal bases of the protein of interest (S3 primer) or the complementary bases downstream (S2 primer). Via PCR the desired linear DNA fragment containing an epitope tag and a selection marker is generated. The PCR product is applied for transformation of competent yeast in order to modify the cells.

For amplification AccuPrime™ *Pfx* DNA polymerase and reaction mix were used (Tab.2) The following protocol was used (Tab.3). The annealing temperature was optimized by using/ a 2-step touch-down PCR with AccuPrime™ *Pfx* DNA polymerase as well (Tab.4). Therefore 8 different annealing temperatures were tested.

Table 2: Components of a PCR with AccuPrime™ *Pfx* DNA polymerase up to a final volume of 50 µL.

Component	**Volume**
10x AccuPrime™ *Pfx* reaction mix	5 µL
AccuPrime™ *Pfx* DNA polymerase	0.5 µL
primer (10 µM each)	1.5 µL (each)
template (100 ng)	0.5 µL
ddH_2O	to 50 µL

Table 3: Protocol for PCR with AccuPrime™ *Pfx* DNA polymerase.

Cycle	Temperature	Time	Repeat
Initial denaturation	97 °C	5 min	/
Denaturation	97 °C	15 sec	33 x
Annealing	59 °C	30 sec	
Elongation	68 °C	1:45 min	
Final Elongation	68 °C	1 min	/
Store	4 °C		/

Table 4: Protocol for 2-step touch-down PCR with AccuPrime™ *Pfx* DNA polymerase.

Cycle	Temperature	Time	Repeat
Initial denaturation	97 °C	4 min	/
Denaturation	97 °C	1 min	11 x
Annealing	50.2 °C, 55.2 °C	30 sec	
Elongation	68 °C	2 min	
	97 °C	1 min	20 x
	59 °C	30 sec	
	68 °C	2 min	
Final Elongation	68 °C	1 min	/
Store	4 °C	Store	/

2.3.5 Purification of DNA

After amplification of the distinct DNA sequence via PCR the solution contains besides linear DNA the used polymerase, dNTPs, primer and not amplified plasmid DNA. Hence before transformation of cells the DNA was purified with Zymo research DNA Clean & Concentrator™-5 kit. At first 50 µL PCR-product were placed in a 1.5 mL tube containing 250 µL DNA Binding Buffer (1:5). After briefly vortexing the solution is transferred in Zymo-Spin™ columns played in a collection tube. The columns were centrifuged at 13,000 g for 30 sec and der flow-through was discarded. Then 200 µL DNA Wash Buffer was added and the columns were centrifuged again as mentioned above. This washing step is repeated a second time. Finally 20 µL ddH_2O were added in the centre of each column placed in 1.5 mL tubes. After incubation for 1 min at RT the columns were centrifuged again to elute the pure DNA. Then the concentration of DNA was determined using a NanoDrop spectrophotometer.

2.3.6 Transformation of yeast cells

In order to transform competent yeast cells first 20 µL DNA, which previously was amplified by PCR, were placed in 1.5 mL tubes. After addition of 50 µL competent cells the solution was well mixed by trapping with a pipette carefully. Besides 6 volumes of PEG medium were admitted and mixed again. After a 30 min incubation at RT DMSO was added to a final concentration of 10%. The cells were incubated again in water bath for 20 min at 42°C and centrifuged at 500 rpm for 3 min. The pellet was resuspended in 3.0 mL YPD and in case of clonat resistance as selection marker incubated overnight at 30 °C while shacking. Using tryptophan for selection the cells were only incubated 3-4 h. Depending on the inserted selecting gene 100 µL cells were plated on plates containing the appropriate medium and incubated for 3-5 days at 30 °C.

PEG buffer (sterile filter)

100 mM	LiOAc
10 mM	tris/HCl (pH 8.0)
40% (w/v)	PEG 4000
1.0 mM	EDTA/NaOH (pH 8.0)

2.3.7 Colony PCR

To prove the success of a yeast transformation a colony PCR was done. According to that a colony was picked from a plate and resuspended in 20 µL PCR-preparation (Tab.5). For amplification STL_Check (fwd) primer and AK205 primer were used. The following protocol was used (Tab.6).

Table 5: Components of a colony PCR up to a final volume of 20 µL.

Component	**Volume**
10x PCR buffer (–$MgCl_2$)	2 µl
Platinum Taq polymerase	0,08 µL
dNTP mix (10 mM)	0.4 µl
$MgCl_2$ (50 mM)	0.6 µl
Primer	0.4 µl (each)
Template DNA	yeast colony
ddH_2O	to 20 µl

Table 6: Protocol of a colony PCR.

Cycle	Temperature	Time	Repeat
Initial Denaturation	94 °C	2 min	/
Denaturation	94 °C	30 sec	30 x
Annealing	55 °C	30 sec	
Elongation	72 °C	1:45 min	
Final Elongation	72 °C	10 min	/
Storage	4 °C		/

2.4 Microscopy

2.4.1 Fluorescence analyses

For studies on gene level the fluorescence intensity of GFP-tagged Fus1 in the investigated strains is detected upon 1 h stimulation with either NaCl or alpha factor. Therefore cells were incubated overnight in 2 mL SD medium at 30 °C while shacking. On the next day cells were diluted to an OD_{600} of 0.05 and incubated to an OD_{600} of 0.1. Then 1 mL culture of each strain was stimulated with either 100 nM pheromone or 0.2 M NaCl for 1 h while shacking. The measurements were made with Cell-R setup containing a MT20 illumination system. Further a 60x water objective and GFP filters were used. Additionally images of unstimulated cells were made to correct autofluorescence of the yeast cells.

SD medium

6.70 g yeast nitrogen base without amino acids
590 mg amino acid mix
20.0 g Glucose
add ddH_2O to 1 L

2.5 Chemical/ biological material

2.5.1 Yeast strains

sCH47: S288c, Bar1::delta::natNT2, Fus1::eGFP::kanMX

sJJ112: sCH47, Ste11::S243A::HIS

sJJ113: sCH47, Ste11::T596I::TRP

sJJ136: sJJ112, Ste11::T596::TRP

sJJ137: S288c, sst1::delta::natNT2, Ste11::3m-eGFP::kanMX, Ste5::3mCherry::hphNT1, Ste11::S243A::HIS, Ste11::T596::TRP

2.5.2 Bacterial strains

Chemical competent *Escherichia coli* XL Gold	Stratagene
Transformed *Escherichia coli* with pYM34	

2.5.3 Antibodies

AF1018 (Anti-Phospho-ERK1/ERK2)	R&D Systems
Donkey anti goat (800)	LI-COR
Donkey anti mouse (680)	LI-COR
Donkey anti rabbit (680)	LI-COR
Fus3 (yC-19)	Santa Cruz Biotechnology
Hog1 (yC-20)	Santa Cruz Biotechnology
Phosphoglycerate Kinase	Invitrogen
P-p53 MAPK rabbit (4511S)	Cell SignallingTechnology

2.5.4 Chemicals

α-factor	Zymo Research
Acetic acid	Fisher Scientific
Acrylamide (30%)	AppliChem
Agarose	Invitrogen
Ampicillin sodium salt	Gerbu Biotech GmbH
APS	Serva
Blocking Buffer Odyssey	LI-COR
Bradford Reagent	SIGMA Life Science
Bromophenol blue	SIGMA
DMSO	Serva
DTT (2.25 M)	Fluka Sigma Aldrich

EDTA	Gerbu Biotech GmbH
Glass beads (acid washed)	SIGMA Life Science
Glucose	Merck
Glycerol	Merck
Glycine	Carl ROTH
HCl	J. T. Baker
LiOAc	SIGMA
2-Log Ladder (0.1-10.0 kb)	New England Biolabs
β-mercaptoethanol	Carl ROTH
Methanol	SIGMA-ALDRICH
NaOH	J. T. Baker
Newblot PVDF Stripping Buffer Odyssey	LI-COR
Orange G	Carl ROTH
PEG 4000	Fluka SIGMA-ALDRICH
Phosphatase Inhibitor Cocktail 2	SIGMA
Phosphatase Inhibitor Cocktail 3	SIGMA
Precision Plus Protein Dual Color Standards	Bio-Rad
Protease Inhibitor Cocktail EDTA free	Roche
RedSafe	IntronBiotechnology
SDS	Carl ROTH
NaCl	VWR Chemicals
Sorbitol	SIGMA Life Science
Sucrose	J.T. Baker
TEMED	Carl ROTH
Tris base	Carl ROTH
Tris HCl	AppliChem Panreac
Tween 20	Serva

2.5.5 Enzymes

AccuPrime™ *Pfx* DNA Polymerase (2.5 U/µL)	Invitrogen
Platinum Taq DNA Polymerase (5 U/µL)	Invitrogen

2.5.6 Plasmids

pMK20	Euroscarf

2.5.7 Primer

S2 (rev)	reverse complement of 66 bases downstream (including STOP) of sequence of interest followed by 5'-ATCGATGAATTCGAGCTCG-3'
S3 (fwd)	65 bases upstream of ATG (including ATG) of sequence of interest followed by 5'-CGTACGCTGCAGGTCGAC-3'
STL_Check (fwd)	5'-TCGACATCATCTTTGC-3'
AK205 (rev)	reverse complement of S3 primer

2.5.8 Kits

Roti®-Prep Plasmid MINI	Carl ROTH
DNA Clean & Concentrator™ -5 Kit	ZYMO RESEARCH

2.5.9 Technical equipment

Centrifuges:	Eppendorf Centrifuge 5415 R
	Eppendorf Centrifuge 5424
	Eppendorf Centrifuge5810 R
Clean bench:	IBS Integra Biosciences
Incubator:	New Brunswick Scientific Innova 4230 Refrigerated Benchtop Incubator Shaker
Electrophoresis:	Bio-Rad Power Pac
	Bio-Rad Precast gel system
Microscope:	Cell R, Olympus
Shaker:	Eppendorf Thermomixer comfort
	Scientific Industries Vortex Genie 2
Spectrometer:	Beckmann Coulter DU800UV/Vis Spectrophotometer
	NanoDrop ND-1000 PEQLAB Biotechnologie GmbH
Thermocycler:	Eppendorf Mastercycler pro
Water bath:	Köttermann
Western Blot:	Bio-Rad system
Western Blot Imager:	Li-COR Odyssey Imaging System

3. Results

3.1 Analysis of MAPK activity using Western Blots

The main topic of this work is based on Western Blot experiments quantifying the phosphorylation level of both MAPKs Fus3 and Hog1 in wild type, Ste11(T596I), Ste11(S243A) and Ste11(S243A/T596I) strains, investigating the influence of disrupting a negative feedback phosphorylation on S243 or a phosphorylation on T596 in Ste11.

The activity of a MAPK and thereby the signal transduction of a distinct MAPK pathway is dependent on dual phosphorylation of two residues in its activation loop. Yeast expressing Ste11-Wt, Ste11(S243A) Ste11(T596I) and Ste11(S243A/T596I) were subjected to Western Blots with antibodies anti total Fus3, double phosphorylated Fus3, total Hog1 and double phosphorylated Hog1 (see Material and Methods). The MAPK activity then was analysed by normalizing the amount of phosphorylated protein to total protein. In each experiment Wt was compared to the mutant strains. For exact comparison of the strains and to exclude technical variations all samples were transferred to one membrane.

The intensity of each band was analysed with the imaging program Fiji. First, the background was substracted manually and in a second step the integrated density of pMAPK and total MAPK band were measured.

3.1.1 Basal phosphorylation levels of Hog1 and Fus3

Analysis of time-dependent phosphorylation of both Fus3 and Hog1 showed differences of basal phosphorylation level in the mutant strains under unstimulated conditions (Fig.10, 11a, 12a). Therefore the arithmetic mean of the phosphorylated fraction of either Fus3 or Hog1 was compared with each other in unstimulated cells (Fig.10).

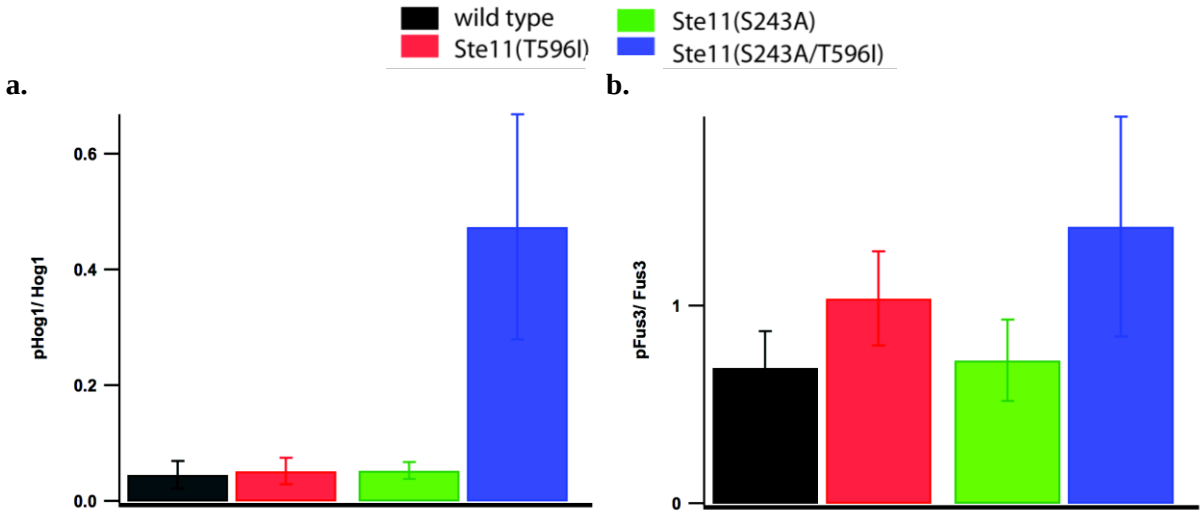

Figure 10: Basal phosphorylation level (0 min) of **a.** Hog1 and **b.** Fus3 to analyse the influence of disturbing a negative feedback to Ste11 under unstimulated conditions. Investigated strains: wild type, Ste11(T596I), Ste11(S243A) and Ste11(S243A/T596I). Mean ratios (± standard error, n=5) of pMAPK per total MAPK

In both cases (pHog1 and pFus3) the Ste11(S243A/T596I) double mutant cells had the highest basal phosphorylation level. Obviously, Hog1 phosphorylation in Ste11(S243A) and Ste11(T596I) cells was not significantly higher than in the Wt strain, whereas in the case of Fus3 the Ste11(T596I) cells exhibited a slight increase. This would explain the observations that in particular the double mutant Ste11(S243A/T596I) exhibits an abnormal morphology. Cells expressing this mutant show far bigger cell size and elongated growth.[40]

3.1.2 Time-courses of Hog1 in the HOG pathway

At first the time profile of the percentage of Hog1 phosphorylation upon NaCl treatment for wild type and the mutant strains is shown in figure 11.

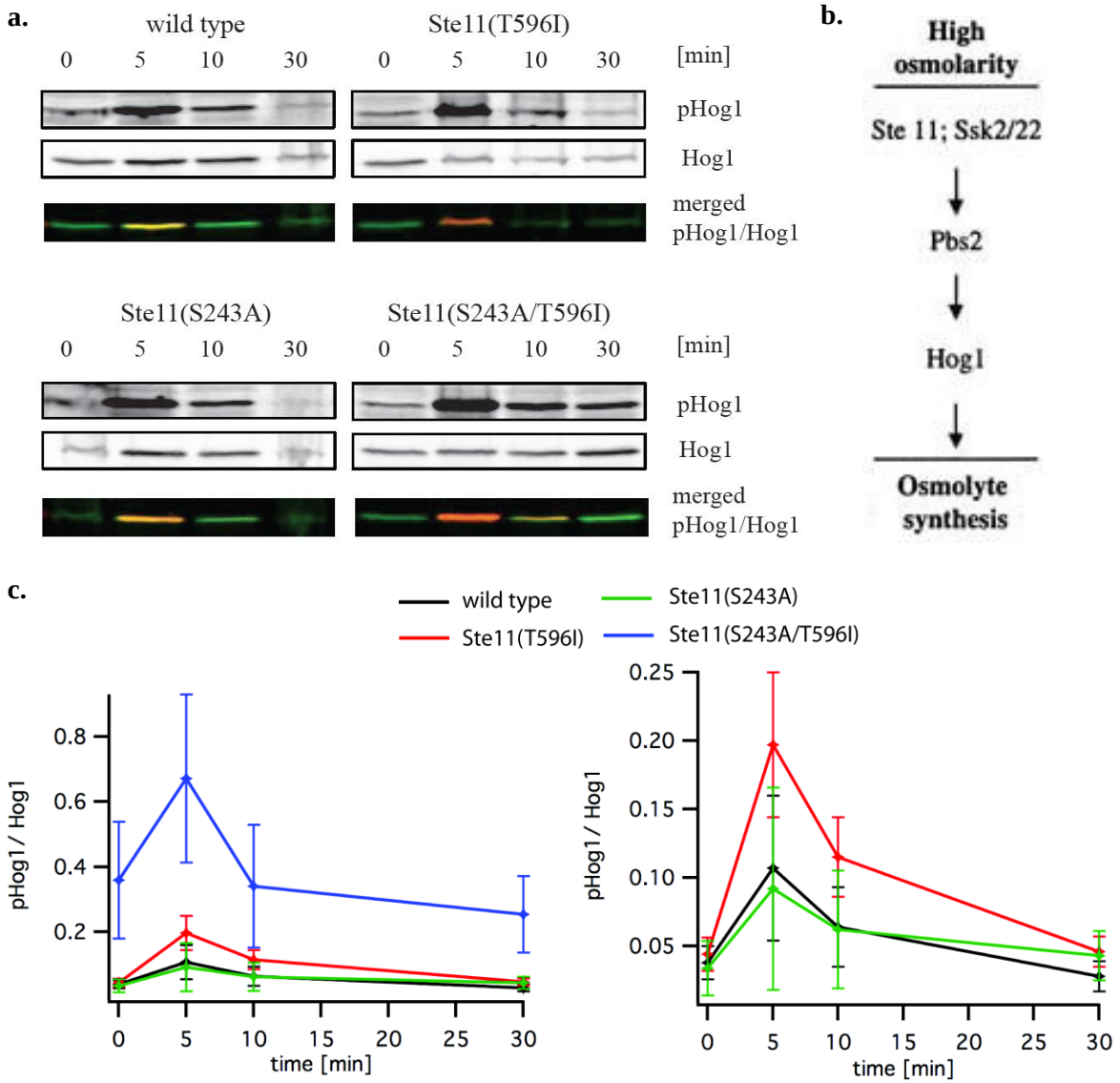

Figure 11: a. Western Blot analysis of pHog1 upon stimulation with 0.2 M NaCl for indicated time points to analyse the influence of disrupting a negative feedback to Ste11. Investigated yeast strains: wild type, Ste11(T596I), Ste11(S243A) and Ste11(S243A/T596I). Uppest panels show phosphorylated Hog1; middle panels show total Hog1; Lowest panels show merged Western blots with total Hog1 in green, pHog1 in red, overlapping bands appear yellow-orange dependent on intensity. **b.** Scheme of analysed pathway. **c.** Corresponding time-course of Hog1 phosphorylation. Mean ratios (± standard error, n=5) of pHog1 per total Hog1. Right graph shows magnification of the lower curves. Hog1 has a molecular weight of 48.9 kDa.

All strains showed a rapid but transient increase of Hog1 activity with a maximum after 5 min stimulation, followed by a drop of pHog1 levels after 10 - 30 min to a basal level as previously described by Macia *et al.*.[43] Obviously, the Ste11(S243A/T596I) double mutant cells exhibited an overall elevated phosphorylation level. Hog1 phosphorylation of Ste11(S243A) and Wt showed the same pattern, but their levels were much lower in comparison to the Ste11(T596I) mutant, which was slightly stronger. Interestingly, the phosphorylation level of Hog1 in Wt, Ste11(S243A) and Ste11(S243A/T596I) cells was almost equal after 10 min compared with its basal level, while the constitutive active Ste11(T596I) strain showed an elevated pHog1 level after 10 min. Only after 30 min the Hog1 phosphorylation level of the Ste11(T596I) strains recovered to its basal pHog1 level. Thus, Hog1 activity decreased after 5 min almost linear up to 30 min in the Ste11(T596I) strain, indicating that the cells needed longer to recover basal Hog1 activity.

3.1.3 Time-courses of Fus3 in the pheromone response pathway

The pheromone-induced pathway was analysed in analogy to the Hog pathway described above (Fig.12, 13).

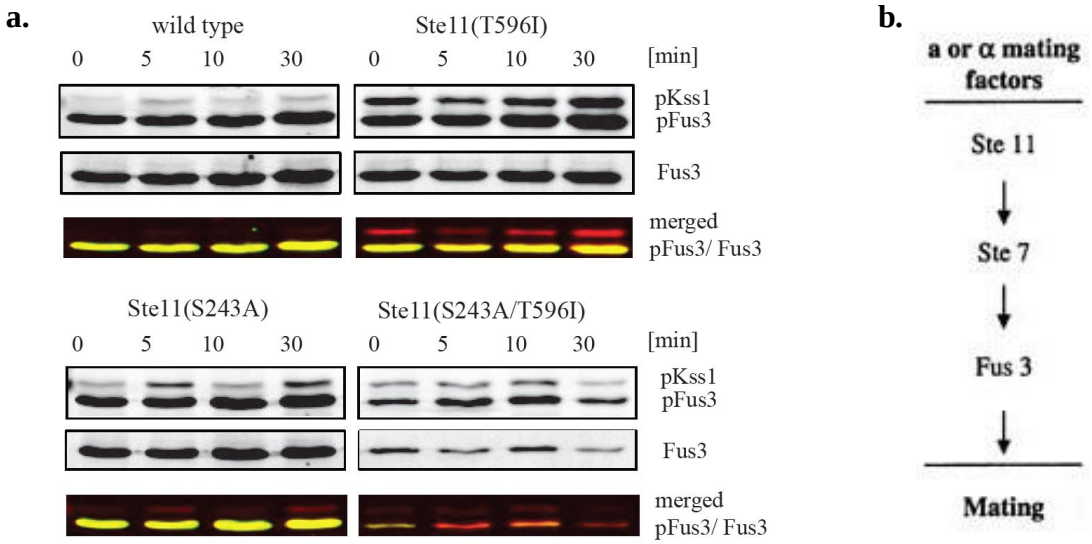

Figure 12: a. Western Blot analysis of pFus3 upon stimulation with 100 nM pheromone for indicated time points to analyse the influence of disrupting a negative feedback to Ste11. Investigated yeast strains: wild type, Ste11(T596I), Ste11(S243A) and Ste11(S243A/T596I). Uppest panels show phosphorylated Fus3; middle panels show total Fus3; Lowest panels show merged Western blots with total Fus3 in green, pFus3 in red, overlapping bands appear yellow-orange dependent on intensity. **b.** Scheme of analysed pathway. Fus3 has a molecular weight of 40.8 kDa.

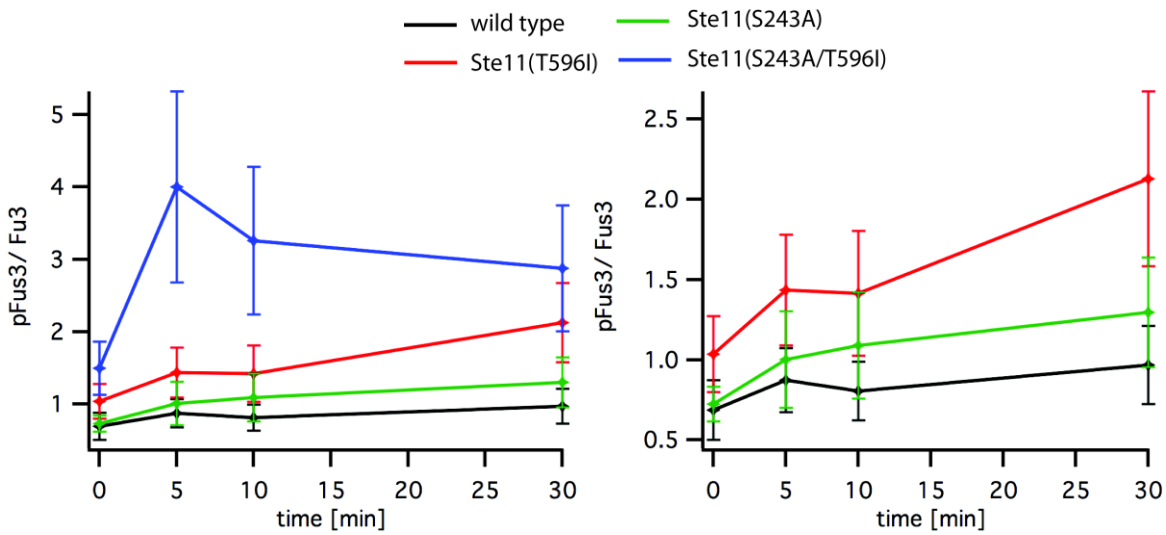

Figure 13: Corresponding time-course of Fus3 phosphorylation in each yeast strain. Mean ratios (±standard error, n=6) of pFus3 per total. Fus3. Right graph shows magnification of the lower curves.

In all strains an increase of pFus3 level after treatment with alpha factor was observed. Obviously, as in the HOG pathway, Ste11(S243A/T596I) cells showed an elevated Fus3 phosphorylation compared to Wt and other mutant strains. Both Ste11(S243A/T596I) double mutant and wild type cells exhibited a slightly transient Fus3 phosphorylation upon pheromone treatment with a maximum at 5 min, whereas the Fus3 phosphorylation level in Ste11(T596I) and Ste11(S243A) strains was graded, but slowly increases up to 30 min.
The ratio of pFus3 in Ste11(S243A/T596I) increased about 2.6-fold after 5 min pheromone treatment relative to basal Fus3 phosphorylation level. In contrast, the amount of pHog1, after 5 min NaCl stimulation, was only 1.8-fold higher than the basal phosphorylation level in Ste11(S243A/T596I). This may indicate that in Ste11(S243A/T596I) cells significantly more Fus3 is activated as response to pheromone treatment than Hog1 upon osmotic stress, whereas in wild type, Ste11(T596I) and Ste11(S243A) cells partially the opposite is observed.
Wt cells exhibited a slight increase of Fus3 phosphorylation after 10 min. In contrast, Fus3 activity in the Ste11(S243A/T596I) strain steadily dropped after 5 min but remained at high phosphorylation level compared to the HOG pathway.

Summarizing, the phosphorylation pattern of Fus3 after pheromone stimulation, an altered Fus3 phosphorylation profile of all mutant strains compared to wild type cells is observed and the Fus3 phosphorylation in wild type cells was at the lowest level relative to the other strains. In contrast, almost the same transient phosphorylation profile of Hog1 upon high external osmolarity in Wt, Ste11(S243A) and Ste11(S243A/T596I) cells was observed; only the Ste11(T596I) strain showed an altered, longer lasting Hog1 phosphorylation. Furthermore, Ste11(T596I) and Ste11(S243A/T596I) cells showed an elevated Hog1 phosphorylation level

at any time, whereas in the Ste11(S243A) strain only slightly more Hog1 was phosphorylated compared to Wt.

3.1.4 Crosstalk between the MAPK pathways in yeast

3.1.4.1 Crosstalk between the HOG and the pheromone response pathway

To investigate leakages potentially prevented by a negative feedback phosphorylation to Ste11 in both the pheromone response and the HOG pathway crosstalk between these pathways was investigated by analysing the phosphorylation pattern of Fus3 after NaCl stimulation and the phosphorylation of Hog after pheromone stimulation.

Figure 14 shows no significant activation of Fus3 through NaCl stimulation in Wt cells. However, it confirms the observation of a generally high basal phosphorylation level of Fus3 in the Ste11(T596I) strains compared to Wt.

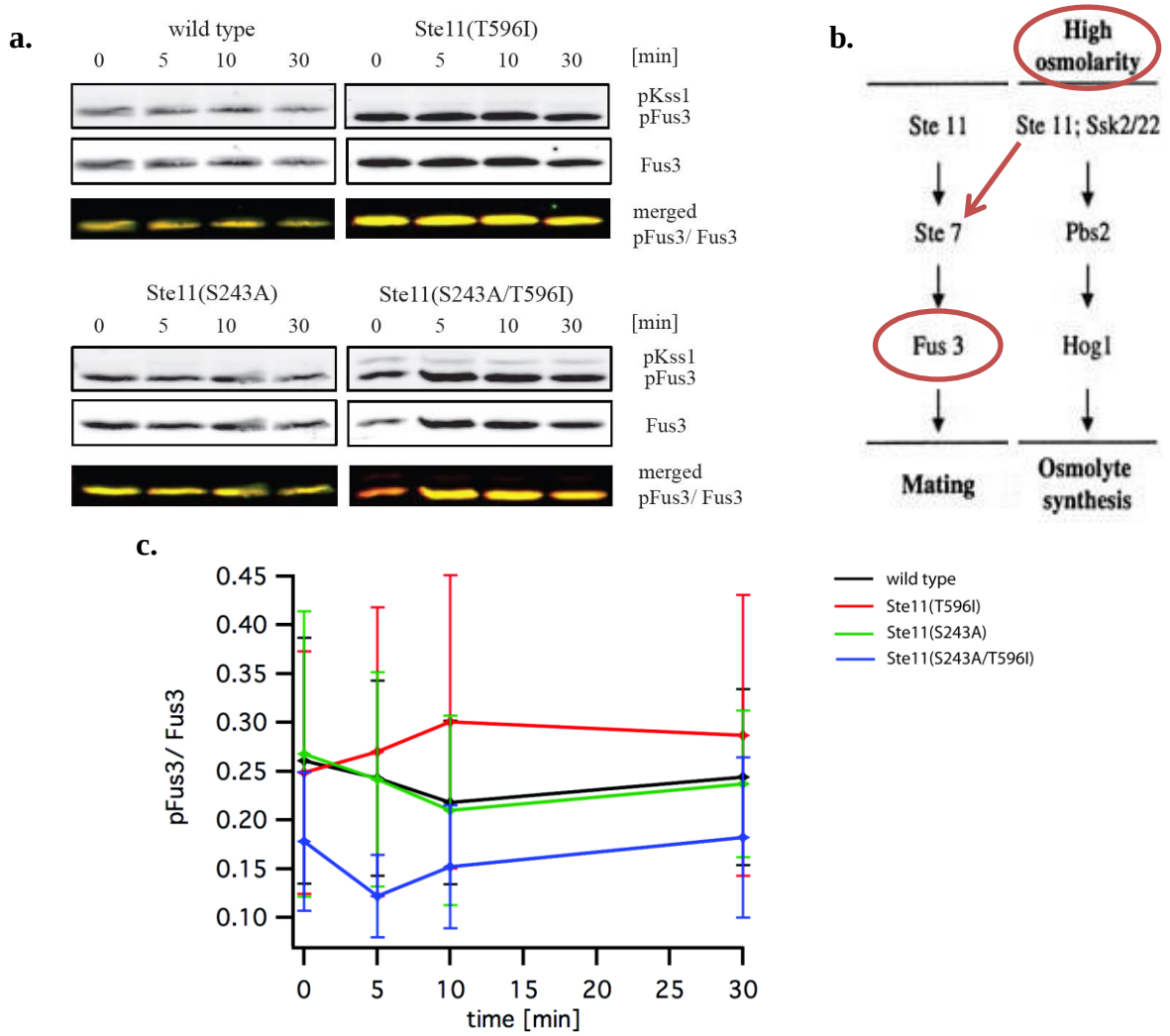

Figure 14: a. Western Blot analysis of pFus3 upon stimulation with 0.2 M NaCl for indicated time points to analyse a potential crosstalk. Investigated yeast strains: wild type, Ste11(T596I), Ste11(S243A) and Ste11(S243A/T596I). Uppest panels show phosphorylated Fus3; middle panels show total Fus3; Lowest panels show merged Western Blots with total Fus3 in green, pFus3 in red, overlapping bands appear yellow-orange dependent on intensity. **b.** Scheme of analysed pathway. **c.** Corresponding time-course of Fus3 phosphorylation in each yeast strain. Mean ratios (±standard error, n=4) of pFus3 per total Fus3.

Unexpectedly, only Ste11(T596I) mutant strains exhibited a stronger phosphorylation of Fus3 than Wt indicating a bleach through between the pathways after disrupting the phosphorylation on T596. In contrast, the Ste11(S243A/T596I) double mutant cells showed the lowest Fus3 phosphorylation level. However, the standard error of each curve in figure 14c is very high so that interpretation of different phosphorylation patterns of each mutant is not possible and requires further analysis.

Analysis of Hog1 activity upon pheromone stimulation revealed significant differences between the yeast strains expressing different forms of Ste11 (Fig.15).

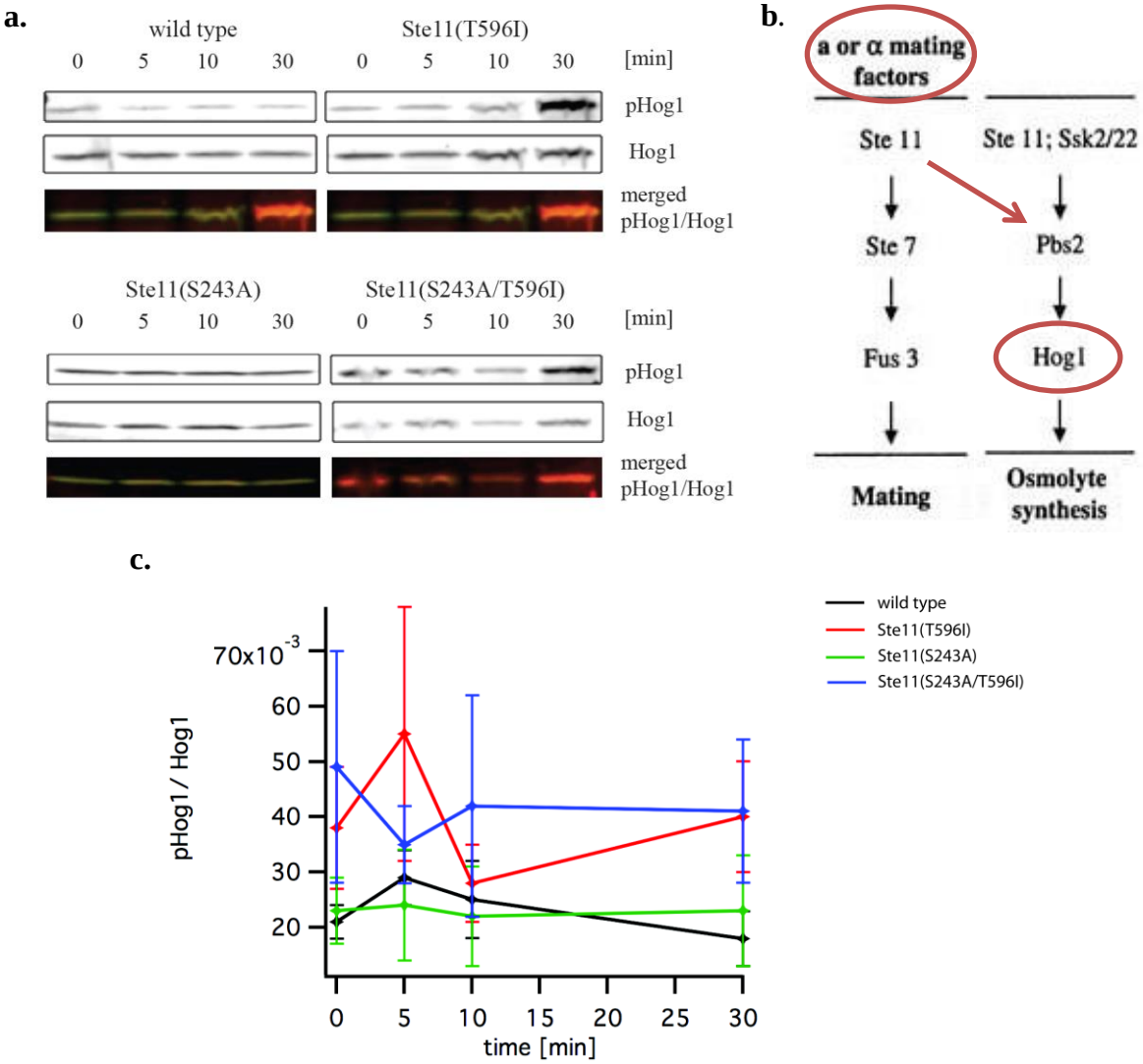

Figure 15: a. Western Blot analysis of pHog1 upon stimulation with 100 nM pheromone for indicated time points to analyse a potential crosstalk. Investigated yeast strains: wild type, Ste11(T596I), Ste11(S243A) and Ste11(S243A/T596I). Uppest panels show phosphorylated Hog1; middle panels show total Hog1; lowest panels show merged Western Blots with total Hog1 in green, pHog1 in red, overlapping bands appear yellow-orange dependent on the intensity. **b**. Scheme of the analysed pathway. **c.** Corresponding time-course of Hog1 phosphorylation in each yeast strain. Mean ratios (± standard error, n=5) of pHog1 per total Hog1.

In the Wt strain Hog1 was slightly activated after stimulation with alpha factor, whereas Ste11(S243A) strains exhibited no increase of Hog1 phosphorylation after pheromone treatment. Both Ste11(S243A/T596I) and Ste11(T596I) cells showed elevated levels of pHog1 and reacted stronger than Wt and Ste11(S243A) cells upon pheromone stimulation. Unfortunately, the standard error of each curve is again very high so that interpretation of different phosphorylation patterns of each mutant is not possible and requires further analysis.

3.1.4.2. Crosstalk between HOG/pheromone response and FG pathway

Furthermore the crosstalk from pheromone response pathway to FG pathway and a potential activation of Kss1 (FG pathway) upon high external osmolarity was studied. Therefore phosphorylated Kss1, detectable with the same antibody that was used for ppFus3, per total PGK was measured. PGK was used as an internal control.

At first Kss1 activation upon high external osmolarity was investigated (Fig.16).

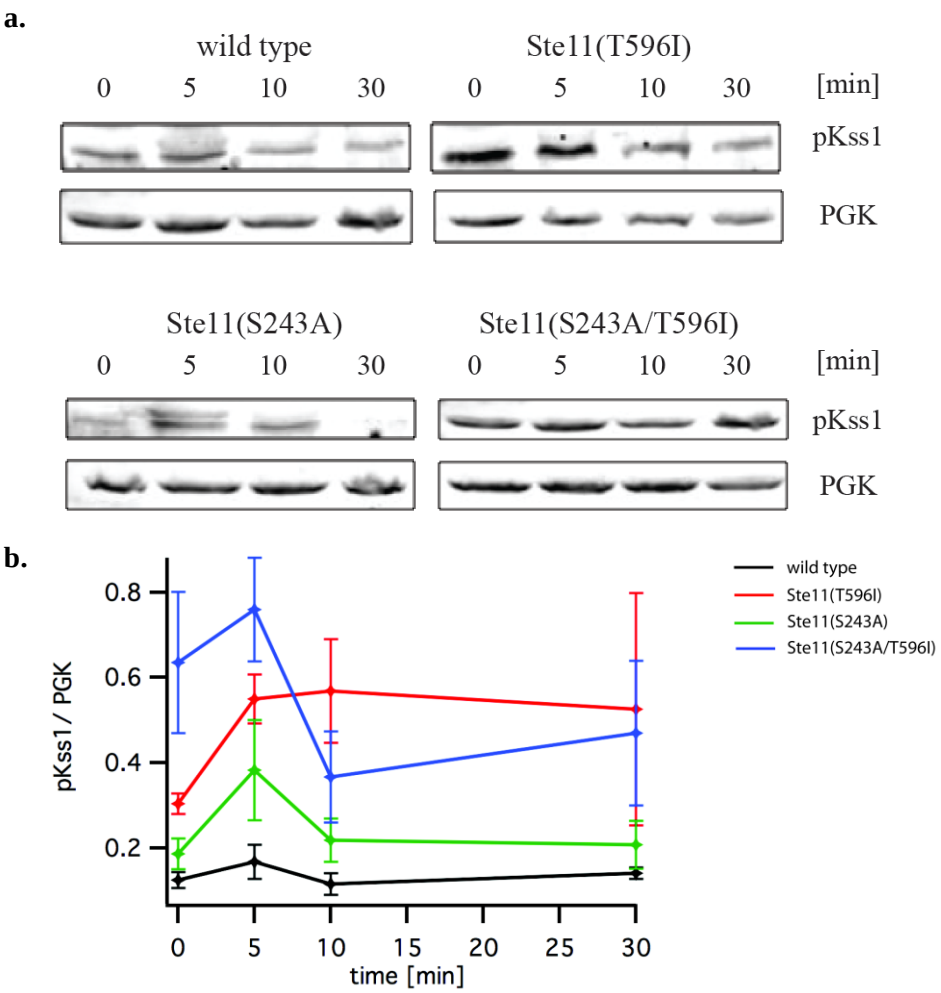

Figure 16: a. Western Blot analysis of pKss1 upon stimulation with 0.2 M NaCl for indicated time points to analyse a potential crosstalk. Investigated yeast strains: wild type, Ste11(T596I), Ste11(S243A) and Ste11(S243A/T596I). Uppest panels show phosphorylated Kss1; lowest panels show total PGK. **b.** Corresponding time-course of Fus3 phosphorylation in each yeast strain. Mean ratios (±standard error, n=4) of pKss1 per total. PGK. Kss1 has a molecular weight of 42.7 kDa.

In wild type cells only a slight increase of Kss1 phosphorylation was observed revealing no or only very little crosstalk between these pathways when the negative feedback phosphorylation is intact. However, disrupting the phosphorylation on T596I in Ste11 leaded to a stronger phosphorylation of Kss1, which reached its maximum after 10 min stimulation and followed a sustained response profile. The Ste11(S243A/T596I) double mutant showed higher phosphorylation of Kss1 as well. Here, the basal phosphorylation was already high followed by a decrease of Kss1 phosphorylation after stimulation, which reached its minimum at 10 min. Phosphorylation of Kss1 in the Ste11(S243A) strain was slightly higher upon NaCl treatment, but the transient phosphorylation profile of Kss1 was similar to the Wt strain.

In comparison to NaCl treatment a different phosphorylation pattern of Kss1 was observed after pheromone stimulation (Fig. 17).

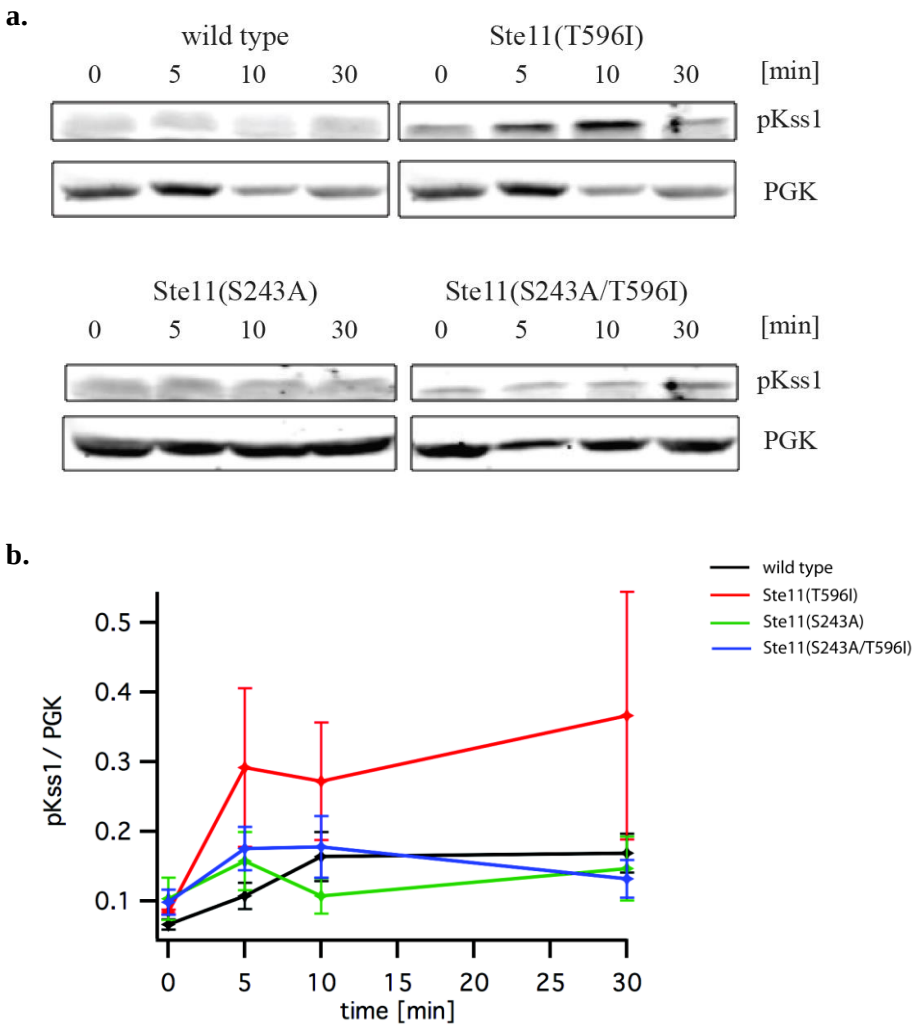

Figure 17: a. Western Blot analysis of pKss1 upon stimulation with 100 nM pheromone for indicated time points to analyse a potential crosstalk. Investigated yeast strains: wild type, Ste11(T596I), Ste11(S243A) and Ste11(S243A/T596I). Uppest panels show phosphorylated Kss1; lowest panels show total PGK. **b.** Corresponding time-course of Fus3 phosphorylation in each yeast strain. Mean ratios (±standard error, n=5) of pKss1 per total PGK.

The Wt, Ste11(S243A) and Ste11(S243A/T596I) double mutant cells showed an increased Kss1 phosphorylation after pheromone treatment. In the Ste11(S243A) mutant Kss1 phosphorylation was more transient than in Wt and Ste11 double mutant. Remarkably, the constitutive active Ste11(T596I) strain revealed a strong Kss1 phosphorylation after 5 min, which slightly decreased up to 10 min and again increased after 10 min. In contrast, the pKss1 level in the other strains was almost on the basal phosphorylation level after 30 min.

Unexpectedly, a significant elevated phosphorylation level of Kss1 in Ste11(S243A/T596I) is not observed, in contrast to previous analysis.

3.3 Microscopy analysis investigating crosstalk

In addition to Western Blot experiments expression of GFP-tagged Fus1, which is expressed by a reporter gene of active Fus3 in the pheromone response pathway, was investigated after treatment with either alpha factor or NaCl for 1 h in the Wt, Ste11(S243A) and Ste11(T596I) strains (Fig.18). These investigations on gene level may confirm the results of Western Blot analysis detecting MAPK phosphorylation status.

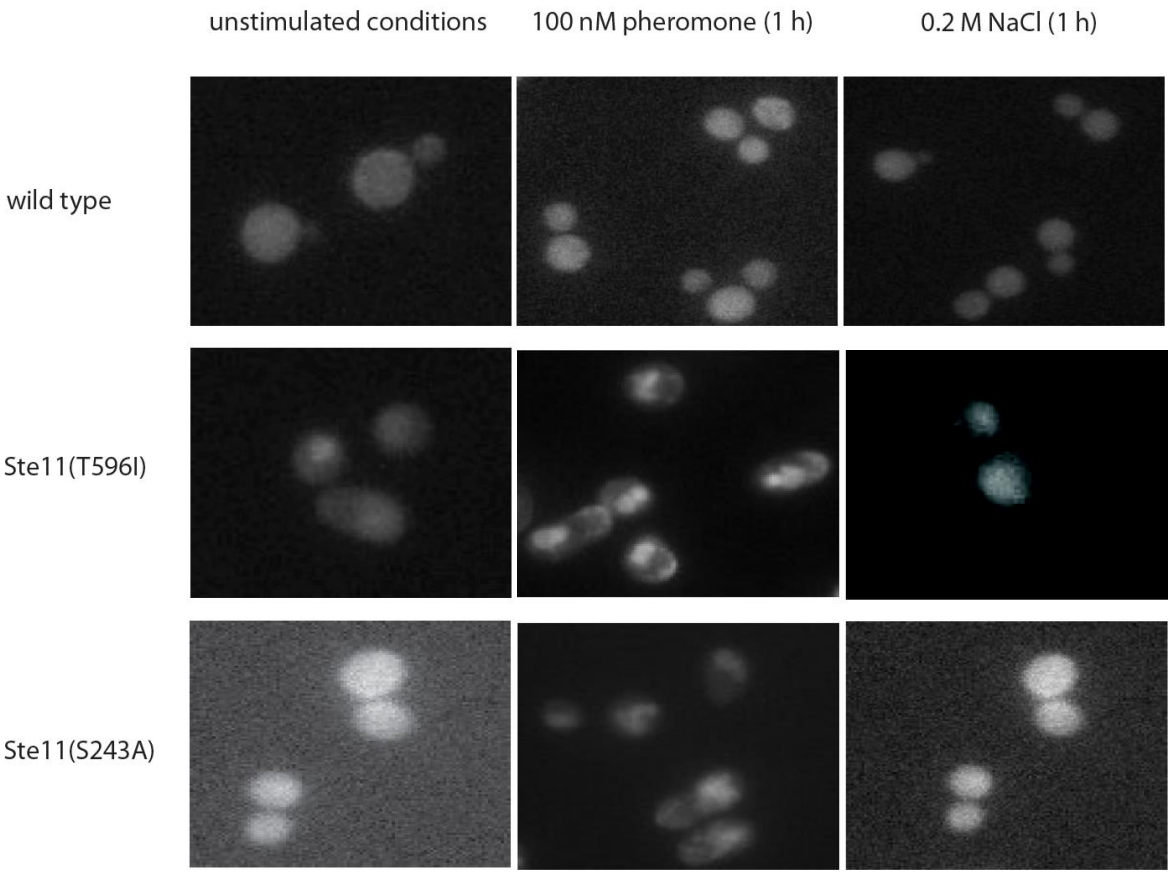

Figure 18: Fluorescence microscopy images for analysis of a potential crosstalk on gene level detecting GFP tagged Fus1, which is a target of pheromone response pathway. All strains measured under unstimulated conditions (left), 1 h after pheromone treatment (middle) and 1 h after NaCl treatment (right). Upper pannels show the wild type strain; middle panels show the Ste11(T596I) strain; low panels show the Ste11(S243A) strain.

For the Wt strain only a slightly higher fluorescence intensity after pheromone treatment was detected in comparison to unstimulated conditions. In contrast, fluorescence intensity of Wt cells upon high external osmolarity did not differ from unstimulated cells.

However, the Ste11(T596I) strain showed a significant GFP-Fus1 accumulation on the membrane, whereas these cells upon NaCl stimulation exhibited only a slightly higher fluorescence intensity compared to unstimulated Ste11(T596I) cells.

Furthermore, in Ste11(S243A) cells GFP-Fus1 accumulation on the membrane was also observed as response to mating signal, but compared to the Ste11(T596I) strain fluorescence intensity seems to be lower. GFP-Fus1 expression upon osmotic stress was not induced in Ste11(S243A) cells as well.

However, these microscopy investigations only indicate an altered GFP-Fus1 expression upon each stimulus and it needs further analysis to quantify the results.
Unfortunately, the Ste11(S243A/T595I) double mutant cells often disappear the GFP-tag, so that is was not possible to measure these cells.

4. Discussion

4.1 Basal Hog1 phosphorylation level

Analysis of the phosphorylation status of Hog1 under unstimulated conditions showed a significant elevated basal pHog1 level in the Ste11(S243A/T596I) strain compared to the other strains. In contrast, in Ste11(S243A) and Ste11(T596I) cells Hog1 activity was only slightly higher in the absence of a stimulus relative to Wt. These findings are consistent with the observations of MAPK activity in other Ste11 dependent pathways, like pheromone response or FG pathway. Here the Ste11(S243A/T596I) strain exhibits a generally elevated activity of the corresponding MAPK.[40]

The observation that the S243A mutation in Ste11 does not alter basal Hog1 phosphorylation is expected because S243 is located in the Ste5 binding site of Ste11, which is not involved in the HOG pathway.

Thus, the effects on basal Hog1 phosphorylation in Ste11(S243A) and Ste11(T596I) cells are not only additive in Ste11(S243A/T596I) double mutant cells. In fact, replacement of two phosphorylation sites in Ste11 to non-phosphorylatable amino acids may per se has an effect on the conformation of Ste11, which causes a higher Ste11 activity and consequently a higher Hog1 phosphorylation under unstimulated conditions. This would explain the significantly higher basal Hog1 phosphorylation in Ste11(S243A/T596I), whereas only a slightly elevated basal pHog1 level in Ste11(S243A) and Ste11(T596I) was observed. Another theory interpreting the exponential elevated Hog1 phosphorylation level in Ste11(S243A/T596I) cells proposes that S243 and T596 in Ste11 are cooperative phosphorylation sites. In that case, in Wt cells, a yet unknown kinase is able to bind phosphorylated S243 and then phosphorylates T596, which leads to an inhibition of Ste11 activity. Mutations in both phosphorylation sites of Ste11 then would result in an elevated Hog1 phosphorylation level as observed in Ste11(S243A/T596I) double mutant cells.

The detection of a basal Hog1 phosphorylation even in wild type cells was expected and is consistent with recent studies of Macia et al.[43] They assumed that the ability of a fast response to small changes in external osmolarity (such as 0.2 M NaCl) is caused through a high basal phosphorylation of Hog1 initiated via the SLN1 branch even in the absence of stress. Further they indicated the contribution of several negative feedback mechanisms, like phosphatases, consistently counteracting through that high basal signal transduction to down-regulate the pathway to a non-detectable output under unstimulated conditions. Mathematical

analysis showed that this negative feedback might be more efficient by down-regulating components upstream of Hog1 and not autoinhibition of Hog1.[44]

Obviously, the balance of highly active SLN1 branch and the counteracting feedback mechanisms seems to be disturbed in Ste11(S243A/T596I) cells resulting in a higher basal pHog1 level. It is possible that not only phosphatases are involved in this interplay ensuring a low basal Hog1 activity but also other yet unidentified feedback mechanisms. This is consistent with computational analysis (Hao *et al*., 2007) assuming that inactivation in general is more effective via inhibition of the target than by activating an inhibitor (like phosphatases).[44]

4.2 Basal Fus3 phosphorylation level

In both unstimulated Ste11(S243A/T596I) and Ste11(T596I) cells a higher Fus3 activity is observed compared to Wt. These findings may explain the elongated morphology and the elevated Fus1 expression in Ste11(S243A/T596I) double mutant cells, even in the absence of pheromone.[40] In contrast, the Ste11(S243A) strain exhibits only a slightly higher pFus3 level under unstimulated conditions. These observations indicate that the stronger binding of Ste5 to Ste11, as caused in the Ste11(S243A) strain, leads only to an slightly elevated pFus3 level, whereas a constitutive active Ste11(T596I) is responsible for a significantly higher Fus3 activity. A combination of both, stronger signal transduction via Ste5-Ste11 binding and a more active Ste11 causes a synergy of the effects, explaining the elevated basal Fus3 phosphorylation in the Ste11(S243A/T596I) double mutant strain.

Thus, Fus3 phosphorylation of S243 in Ste11 combined with phosphorylation of Ste11(T596) ensures a low basal Fus3 activity under unstimulated conditions.

4.3 Time-courses of Hog1 in the HOG pathway

The observed phosphorylation pattern of Hog1 upon osmotic stress showed a distinct transient Hog1 phosphorylation with a maximum at 5 min for all four strains. In wild type these findings are consistent with current literature (Schaber *et al*.).[45] In case of the mutant strains an overall more sensitive response to high external osmolarity in Ste11(T596I) and Ste11(S243A/T596I) cells is expected as a result of a constitutive active Ste11.

As expected Ste11(T596I) and Ste11(S243A/T596I) cells show a stronger Hog1 phosphorylation after 5 min, compared to wild type cells. This indicates that phosphorylation of T596 in Ste11 ensures a desensitisation of the HOG system. The strongest Hog1 phosphorylation in Ste11(S243A/T596I) cells is on one hand the result of the inherently high

basal pHog1 level and on the other hand may confirm the theory of an cooperative kinase binding on one of the phosphorylation sites.
In contrast, in Ste11(S243A) cells no significant altered phosphorylation pattern compared to wild type strain is observed. These results were highly expected considering that S243 is located in the Ste11 binding domain of the Ste5 scaffold protein. Hence disturbing the negative feedback from Fus3 to Ste11 has no influence on the HOG pathway using Pbs2 as a scaffold proteins and not Ste5.
Taken together phosphorylation of T596 leads to a desensitising of the HOG pathway, whereas phosphorylation of S243 by Fus3 does not appear to inhibit Ste11 activity and in turn the signal transduction.

Unexpectedly, in Ste11(S243A/T596I) cells the Hog1 phosphorylation level after 10 min is almost equal to basal Hog1 activity, which is also observed in wild type and Ste11(S243A) cells. Consequently this rapid drop of Hog1 activity is not a result of feedback phosphorylation of either S243, T596 or of both. But these observations may demonstrate the interplay of the different mechanisms to down-regulate the HOG pathway provoked through (excessive) Hog1 activity. As described before pHog1 causes an increased activation of Ptp2 resulting in dephosphorylation of dual-phosphorylated Hog1. Additionally, active Hog1 may be able to down-regulate the SHO1 branch, which is responsible for long term osmoadaption (over a period of 30 min after stimulation) in case of stimulation with low concentrations of NaCl ($\leq$ 0.2 M).[45] Indeed the main fraction of Hog1 is activated via the SNL1 branch (about 80 %), but Schaber *et al.* discovered that the ratio of pHog1 via SLN1 branch is dropped to about 50 % almost after 10 min, whereas the maximal amount of Hog1 via the SHO1 branch (40%) is phosphorylated after 10 min. Inhibition of the SHO1 branch through overactive pHog1, as caused in Ste11(S243A/T596I) cells, may be initiated earlier and stronger as in Wt. Hence, the rapid and in particular strong decrease of pHog1 in Ste11(S243A/T596I) cells after 5 min of NaCl treatment could be a result of the over-activated negative feedback from highly active pHog1 in the SHO1 branch.

A different phosphorylation pattern of Hog1 is observed for the Ste11(T596I) strain, which steadily decreases after 5 min up to 30 min. It is possible that the elevated Hog1 phosphorylation level is not high enough to generate strong negative feedback from pHog1 to the SHO1 branch, like in Ste11(S243A/T596I) cells. Consequently no additional mechanisms like in Wt cells are activated to down-regulate the HOG pathway, although Ste11 is constitutive active.

4.4 Time-courses of Fus3 in the pheromone response pathway

Previous studies observed a maximal phosphorylation of Fus3 after 3 min of pheromone treatment.[46] In order to compare both pathways with each other the time points of measuring the phosphorylation status of Fus3 were chosen the same as for the Hog1 pathway resulting a less strong Fus3 phosphorylation than after 3 min.

However, as in the HOG pathway the Ste11(S243A/T596I) strain shows a significant altered phosphorylation pattern than Wt. This confirms the general findings of an overall higher MAPK activity in the investigated pathways.

Expectedly, a relatively similar Fus3 phosphorylation profile for Wt and Ste11(T596I) cells is observed, except for a general higher pFus3 level in the mutant cells caused by constitutive active Ste11. These findings suggest that a phosphorylation on T596 in Ste11 is only responsible for a desensitisation of the pathway upon pheromone treatment, as described in HOG pathway.

In contrast, activity of Fus3 in Ste11(S243A) cells steadily increases up to a maximum at 30 min, which may indicate that phosphorylation of S243 by Fus3 ensures a transient mating signal in response to pheromone stimulation. Disturbing the negative feedback phosphorylation on S243 in Ste11 causes a stronger binding of Ste11 and Ste5, which may promote signal transduction over a longer time.

The effects on Fus3 phosphorylation observed in Ste11(S243A) and Ste11(T596I) cells may be combined or even show a cooperative effect in the Ste11(S243A/T596I) double mutant strain. This would explain the obviously higher pFus3 levels and the slightly transient Fus3 activation after 5 min.

In summary the results show a different influence of mutant strains on pheromone response pathway than on Hog1 activity. First, phosphorylation of only S243 in Ste11 has no influence in the HOG pathway, but it may ensure a more transient Fus3 activity in mating pathway, whereas, second, phosphorylation of T596 in Ste11 is required for desensitising both pathways. Third, mutations of both S243 and T596 indicate the existence of several other mechanisms involved in ensuring MAPK dynamic or reveal changes in Ste11 structure, as observed in the HOG pathway. In the pheromone response pathway feedback phosphorylation of both sites in Ste11 is responsible for the typical phosphorylation pattern of active Fus3 upon mating.

4.5 Crosstalk between the MAPK pathways in yeast

4.5.1 Crosstalk between the HOG and mating pathway

Potential crosstalk between the pheromone response and the HOG pathway, which may be prevented via a Ste11 (feedback) phosphorylation (by Fus3), was studied. In that case an elevated phosphorylation of Fus3 upon high external osmolarity in mutant strains but not in wild type cells was expected.

The observations were highly unexpected indicating no crosstalk from induced HOG pathway through pheromone response pathway. Neither Ste11(S243A), Ste11(T596I) nor the Ste11(S243A/T596I) double mutant strain showed an increased pFus3 level upon high osmolarity. However, a Fus3 activity in wild type cells is also not.
Only a generally higher basal phosphorylation level of Fus3 in mutant strains is observed, but no activation of Fus3 upon NaCl stimulation. These findings indicate that crosstalk prevention, from HOG pathway to pheromone response pathway, is not based on the investigated negative (feedback) phosphorylation on Ste11, but perhaps on the use of specific scaffold proteins described by Saito *et al.*.
The observations may confirm the previous findings of O'Rourke *et al.* (1998), which discovered that Δ Hog1 or mutant proteins defective in their activity lead to high levels of crosstalk during high external osmolarity. They further proposed that osmotic stress induces mating through the SHO1 branch requiring Ste20 and Ste50, but not the scaffold protein Ste5. Further crosstalk might be prevented by feedback phosphorylation of Sho1 by Hog1. Reports of Yamamoto *et al.* discovered a feedback phosphorylation of Ste50 by Hog1 down-regulating HOG pathway.

Summing up, the previous work of O'Rourke, the results of Yamamoto *et al.* and the findings that Ste11 mutant strains, which obviously cause a higher Hog1 activity, show no crosstalk, lead to the conclusion that crosstalk preventing of the HOG pathway is arranged upstream of the MAPK module. Presumably, Hog1 feedback phosphorylation of Ste50 in the SHO1 branch ensures pathway insulation, at least through high external osmolarity.

Recent reports of simultaneous stimulation experiments by Nagied *et al.* may confirm this theory describing an additional function of the negative Ste50 phosphorylation by Hog1.[46] They assumed that inhibition of Ste50, which is also acting in the mating pathway, leads consequently to a down-regulation of the mating pathway upon co-stimulation. In addition Hog1 may only dampen mating signalling as long as osmoadaption occurs. These

observations correspond with some theoretical models (Resing *et al*.) emphasising the biological significance of crosstalk inhibition within these two pathways: Adaption to osmotic stress is essential for cell surviving, whereas mating decision requires an appropriate environment.[47] Hence, suppression of other MAPK pathways upon high external osmolarity is a biological inevitable process supporting osmoadaption of the cell. After re-establishing normal cell volume the HOG pathway is inhibited by several feedback mechanisms and in case of co-stimulation the mating process can occur.

Summarizing these reports, crosstalk prevention can be described as a complex interplay of numerous feedback inhibition mechanisms demonstrating the dynamics of the MAPK pathways (Fig.19).

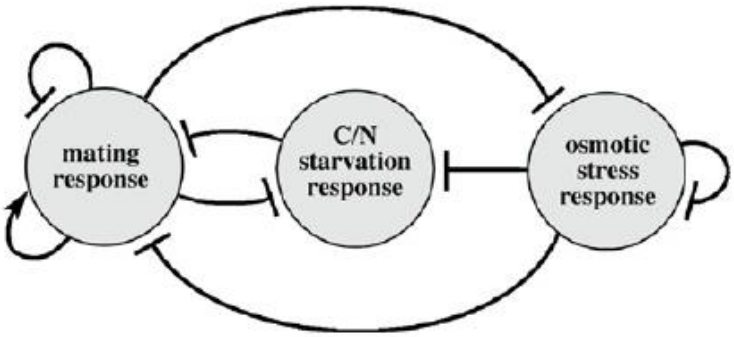

Figure 19: Scheme of several feedback regulation MAPK pathways in yeast illustrating the dynamics of MAPK pathways. Pheromone response pathway is positively and negatively regulated by itself; down-regulates the HOG and the C/N starvation response pathway and is down-regulated by the HOG pathway. HOG pathway down-regulates itself and the C/N starvation response pathway.[47]

Interestingly, an activation of Hog1 upon pheromone treatment in the Ste11(T596I) and Ste11(S243A/T596I) strains could be observed. These findings would rebut the reports of Saito *et al.* claiming that both pathways are insulated from each other caused by the involvement of specific scaffold proteins (e.g. Ste5).

The observations indicate that disturbing the phosphorylation on T596 in Ste11 leads to a leakage in the pheromone response pathway through the HOG pathway. The rapid decrease of phosphorylated Hog1 in Ste11(T596I) cells after 5 min could be explained with the activation of several feedback mechanisms from active Hog1, e.g. increased phosphatase activity.

In contrast, Ste11(S243A) and wild type strain exhibit no significant Hog1 activity upon pheromone treatment, which could be a result of using specific scaffold proteins as described by Saito *et al.*. However, insulation of the pheromone response pathway occurs not only

through different scaffold proteins but is also ensured by the investigated phosphorylation to T596 in Ste11. Consequently both Sho1 and Ste50 are not required for partial signal transduction via Ste11 and Pbs2 and in turn Hog1 phosphorylation.

The results relating to a potential crosstalk in mutant strains are summarized in figure 20.

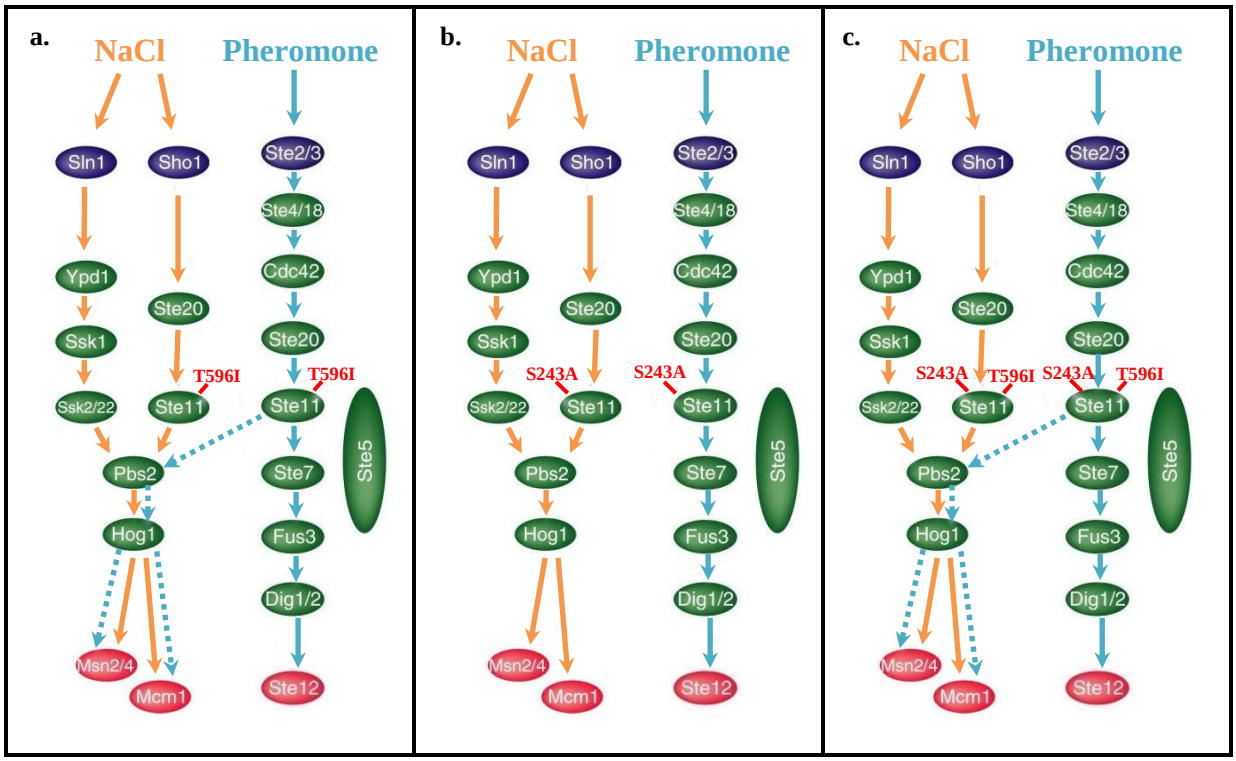

Figure 20: Illustration of potential crosstalk within the pheromone response and HOG pathway in the investigated mutant strains upon pheromone stimulation or high external osmolarity. **a.** the Ste11(T596I) strain shows Hog1 activation upon pheromone treatment via a crosstalk probably from the shared MAPKKK Ste11; Fus3 activity upon osmotic stress was not observed. **b.** the Ste11(S243A) strain shows no Hog1 activation upon pheromone treatment and no activation of Fus3 upon osmotic stress indicating no crosstalk prevention from Ste11-S243. **c.** the Ste11(S243A/T596I) strain shows Hog1 activation upon pheromone treatment via a crosstalk probably from the shared MAPKKK Ste11; Fus3 activity upon osmotic stress was not observed. Adapted from Steffen *et al.* .[48]

4.5.2 Crosstalk between the HOG and the FG pathway

In addition Kss1 phosphorylation patterns upon stimulation with NaCl were investigated expecting a higher ratio of crosstalk in mutant strains.

Wt cells reveal no significant activation of Kss1 upon high external osmolarity indicating no leakage from HOG pathway to FG pathway. Interestingly, in both Ste11(S243A) and Ste11(T596I) Kss1 shows a higher phosphorylation level upon NaCl stimulation. In contrast to the transient Kss1 activation in the Ste11(S243A) strain the Kss1 activation in Ste11(T596I) is stronger and has a maximum at 10 min after NaCl treatment. Unfortunately, the standard error of the curves of mutant strains are very high so that interpretation need

further analysis. Remarkably, the mutant strains exhibit a higher basal Kss1 phosphorylation compared to Wt. These findings confirm the observations of a general stronger basal MAPK activity in all three investigated pathways, in particular for Ste11(T596I) and Ste11(S243A/T596I).

4.5.3 Crosstalk between the pheromone response and the FG pathway

Additionally, a leakage in mating pathway to FG pathway was analysed expecting an elevated pKss1 level in the Ste11(T596I) and Ste11(S243A/T596I) strains relative to Wt cells.

Indeed, Ste11(T596I) cells show a significant elevated Kss1 activity upon pheromone treatment, but Ste11(S243A/T596I)-Kss1 activity is not significant higher compared to Wt. However, current literature describes a leakage from mating pathway to FG pathway, even in Wt cells, which is also observed in this work.

As previously reported by Saito *et al.* even in Wt cells a Kss1 activation upon pheromone treatment is observed, although the mating pathway uses the specific scaffold protein Ste5. According to that a higher Kss1 phosphorylation in the Wt strain upon pheromone stimulation was expected. Further reports (Sabbagah *et al*.) indicate that Kss1 activation upon mating signal is required for inducing gene expression.[49] Thus, Fus3 and Kss1 work together in response to pheromone treatment, but active Fus3 may be able to limit the extent of the Kss1 activation upon mating. With regards to Saito *et al.* transient activation of Kss1 upon mating is based on a kinetic insulation strategy postulating cross talk preventing via different dose-responses and time-courses.
Actually the time-courses of pFus3 and pKss1 upon pheromone treatment confirm the kinetic insulation strategy, at least in the Wt and Ste11(S243A) strains. They show a more or less steady increase of Fus3 activity, whereas Kss1 phosphorylation is more transient.
An elevated Kss1 activity in Ste11(T596I) cells is a result of constitutive active Ste11, which may promote a crosstalk ratio by disturbing the balance between transient Kss1 activation and Fus3 activity.

Furthermore, a extenuated Kss1 activation in Ste11(S243A) cells was expected. Mutation of S243 in Ste11 leads to an increased affinity of Ste5 binding, which promotes mating signal, but has no function in FG pathway. According to that it is possible that Ste11(S243A)-Ste11 is frequently associated with Ste5 resulting in a reduced crosstalk rate. However, this does not explain the more transient response of Kss1 in the Ste11(S243A) strain.

The observations in the Ste11(S243A/T596I) double mutant suggest that the effects of S243A and T596I in Ste11 partially balance each other. Further, it is possible that the increased affinity of Ste5 to bind Ste11, which is caused through S243A mutation, leads to a reduced availability of catalytic free Ste11. Thereby, signal transduction of Ste5-independent pathways, like FG pathway, is decreased. However, this explanation would be in conflict with the observation in the HOG pathway, where the Ste11(S243A/T596I) double mutant strain exhibits a stronger signal transduction, although Ste5 is not required.

4.6 Microscopy analysis of crosstalk

In addition to the Western Blot analysis investigating the MAPK phosphorylation status the signal transduction of the pheromone-induced pathway was analysed on genomic transcription level.

With regards to the results mentioned above a higher GFP-Fus1 expression upon pheromone stimulation in the mutant strains, at least in Ste11(T596I) cells, was expected. In contrast no significant activation of mating genes upon high external osmolarity was expected neither in Wt nor in Ste11(S243A) and Ste11(T596I) indicating pathway insulation.

The detected GFP-Fus1 expression in Ste11(T596I) cells confirms the observations on Western Blots indicating not only an elevated Ste11(T596I)-Fus3 activity but also stronger activation of mating genes, in particular FUS1 gene. Already after 60 min incubation Ste11(T596I) cells show a significant GFP-Fus1 accumulation at the membrane, whereas Wt cells only show a slightly but not significant overall increase of fluorescent intensity. Relating to recent findings significant GFP-Fus1 expression in Wt is reached after 90 min of pheromone treatment, which is up to one cell cycle. Therefore GFP-Fus1 accumulation on the membrane in the Wt strain only can be detected after 90 min. This suggests that mating signal is transduced stronger in Ste11(T596I) cells leading to an earlier response on gene level. Expectedly, the Ste11(S243A) strain also exhibits stronger GFP-Fus1 expression and its recruitment to the plasma membrane. But fluorescent intensity might be slightly lower than in the Ste11(T596I) strain. These findings are consistent with the results of investigated Fus3 activity indicating that in particular phosphorylation of T596 in Ste11 leads to an extenuated mating response. Additionally it confirms several studies suggesting that Ste5 binding on Ste11 promotes mating signal transduction.

5. Outlook

The results of this bachelor thesis give further hints on the existence of a negative feedback phosphorylation on S243 in Ste11 and a phosphorylation of T596 influencing three MAPK pathways in yeast. Furthermore it was shown that disturbing this phosphorylation on Ste11 leads to a leakage of several pathways, at least on MAPK level.

Consequently, these results should be investigated on gene level. Due to the short time of the bachelor thesis it was not possible to tag STL1 gene, which is expressed in response to high external osmolarity. Thus, some FACS studies investigating expression of GFP-Fus1 and mCherry-STL1 in Ste11 mutant cells upon either pheromone stimulation or osmotic stress should be made. Furthermore, the influence of disturbing the phosphorylation on Ste11 on other MAPK pathways in yeast should be analysed. Presumably, the double mutant Ste11(S243A/T596I) strain has also effects on the cell wall integrity and the spore wall assembly MAPK pathways, despite both are independent on Ste11. This was assumed by the fact that Ste11 double mutant cells exhibit an abnormal, elongated morphology.

In addition further analysis should focus on the origin of the phosphorylation targeting on Ste11-T596.

6. References

[1] Chen, RE. and Thorner, J. Function and regulation in MAPK signaling pathways: lessons learned from the yeast Saccharomyces cerevisiae. *Biochim Biophys Acta* **1773**, 1311-1340 (2007).

[2] Martín, H., Flández, M., Nombela, C. and Molina, M. Protein phosphatases in MAPK signalling: we keep learning from yeast. *Molecular microbiology* **58**, 6-16 (2005).

[3] v. Dorgen, F., O'Rourke. SM., Stucke, V.M., Jaquenoud, M., Neiman, AM. and Peter, M. Phosphorylation of the MEKK Ste11p by the PAK-like kinase Ste20p is required for MAP kinase signalling *in vivo*. *Current biology* **10**, 630-639 (2000).

[4] Pearson, G., Robinson, F., Beers Gibson, T., Xu, B., Karandikar, M., Berman, K. and Cobb, MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine reviews* **2**, Vol. 22 (2001).

[5] Saito, H. Regulation of cross-talk in yeast MAPK signaling pathways. *Current opinion in microbiology* **13**, 677-683 (2010).

[6] Pierce, KL and Lefkowitz, RJ. Classical and new roles of β-arrestins in the regulation of G-protein-coupled receptors. *Nature reviews neuroscience* **2**, 727-733 (2001).

[7] Wu, C., Leberer, E., Thomas, DY. and Whiteway, M. Functional characterization of the interaction of Ste50p with Ste11p MAPKKK in *Saccharomyces cerevisiae*. *Molecular biology of the cell* **10**, 2425-2440 (1999).

[8] Saito, H. and Tatebayashi, K. Regulation of the osmoregulatory HOG MAPK cascade in yeast. *Journal of biochemistry* **136**, 267-272 (2004).

[9] Bardwell, L., Cook, JG., Chang, EC., Cairns, BR. and Thorner, J. Signaling in the yeast pheromone response pathway: specific and high-affinity interaction of the mitogen-activated protein (MAP) kinases Kss1 and Fus3 with the upstream MAP kinase kinase Ste7. *Molecular and cellular biology* **16**, 3637-3650 (1996).

[10] Saito, H. and Posas, F. Response in hyperosmotic stress. *Genetics* **192**, 289-318 (2012).

[11] Hohmann, S. Osmotic stress signalling and osmoadaption in yeasts. *Microbiology and molecular biology reviews* **66**, 300-372 (2002).

[12] Raitt, DC., Posas, F. and Saito, H. Yeast Cdc41 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway. *The EMBO journal* **19**, 4623-4631 (2000).

[13] O'Rourke, SM. and Herskowitz, I. A third osmosensing branch in *Saccharomyces cerevisiae* requires the Msb2 protein and functions in parallel with the Sho1 branch. *Molecular and cellular biology* **22**, 4739-4749 (2002).

[14] Tatebayashi, K., Tanaka, K., Yang, HY., Yamamoto, K., Matsushita, Y., Tomida, T., Imai, M. and Saito, H. Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. *The EMBO journal* **26**, 3521-3533 (2007).

[15] Posas, F., Wurgler-Murphy, SM., Maeda, T., Witten, EA., Thai, TC. and Saito, H. Yeast Hog1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the Sln1-Ypd1_Ssk1 "two-component" osmosensor. *Cell* **86**, 865-875 (1996).

[16] Eikel, I., Sulea, T., Jansen, G., Kowalik, M., Minailiuc, O., Cheng, J., Harcus, D., Cygler, M., Whiteway, M. and Wu, C. Binding the atypical RA domain of Ste50p to the unfolded Opy2 cytoplasmic tail is essential for the high-osmolarity glycerol pathway. *Molecular biology of the cell* **20**, 5117-5126 (2009).

[17] Klipp, E. and Liebermeister, W. Mathematical modeling of intracellular signaling pathways. *BMC neuroscience* **7** (2006).

[18] Alepuz, PM., de Nadal, E., Zapater, M., Ammerer, G. and Posas, F. Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA pol II. *The EMBO journal***. 22**, 2433-2442 (2003).

[19] Ferreira, C., van Voorst, F., Martins, A., Neves, L., Oliveira, R., Kielland-Brandt, M.C., Lucas, C. and Brandt A. A member of the sugar transporter family, Stl1p is the glycerol/H^+ symporter in *Saccharomyces cerevisiae*. *Molecular biology of the cell* **16**, 2068-2076 (2005).

[20] Stranger, K., Gorelik, M. and Davidson, AR. Yeast adaptor protein, Nbp2, is conserved regulator of fungal Ptc1 phosphatases and is involved in multiple signalling pathways. *The journal of biological chemistry* **26**, 22133-22 2012).

[21] Mapes, J. and Ota, IM. Nbp2 targets the Ptc1-type 2C Ser/Thr phosphatase to the HOG MAPK pathway. *The EMBO journal* **23**, 302-311 (2004).

[22] Yamamoto, K. Tatebayashi, K., Tanaka, K. and Saito, H. Dynamic control of yeast MAP kinase network by induced association and dissociation between the Ste50 scaffold and the Opy2 membrane anchor. *Molecular cell* **40**, 87-98 (2010).

[23] O'Rourke, SM and Herskowitz, I. The Hog1 MAPK prevents cross talk between HOG and pheromone response MAPK pathway in Saccharomyces cerevisiae. *Genes & development* **18**, 2874-86 (1998).

[24] Hao, N., Zeng, Y., Elston, TC. and Dohlman, HG: Control of MAPK specificity by feedback phosphorylation of shared adaptor protein Ste50. *The journal of biological chemistry* **49**, 33798-802 (2008).

[25] Warmka, J., Hannerman, J., Lee, J., Amin, D. and Ota, I. Ptc1, a type 2C Ser/Thr phosphatase, inactivates the HOG Pathway by dephosphorylating the mitogen-activated protein kinase Hog1. *Molecular and cellular biology* **1**, 51-60 (2001).

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

[27] McClean, MN., Mody, A., Broach, JR. and Ramanathan, S. Cross-talk and decision making in MAP kinase pathways. *Nature genetics* **39**, 409-413 (2007).

[28] Shock, TR., Thompson, J., Yates III, JR. and Madhani, HD. Hog1 mitogen-activated protein kinase (MAPK) interrupts signal transduction between the Kss1 MAPK and the Tec1 transcription factor to maintain pathway specifity. *Eukaryotic cell* **8**, 606-616 (2009).

[29] Witzel, F., Maddison, L. and Blüthgen, N. How scaffolds shape MAPK signalling: what we know and opportunities for systems approaches. *Frontiers in physiology* **3**, 475 (2012).

[30] Elion, EA. Pheromone response, mating and cell biology. *Current opinion in microbiology* **3**, 573-581 (2000).

[31] Bradwell, L. A walk-through of the yeast mating pheromone response pathway. *Peptids* **26**, 339-350 (2005).

[32] Wiget, P., Shimada, Y., Butty, AC., Bi, E. and Peter, M. Site-specific regulation of the GEF Cdc24p by the scaffold protein Far1p during yeast mating. *The EMBO journal* **23**, 1063-1074 (2004).

[33] Malleshaiah, MK., Shahrezaei, V., Swain, PS. and Michnick, SW. The scaffold protein Ste5 directly controls a switch-like mating decision in yeast. *Nature* **465**, 101-105 (2010).

[34] v. Drogen, F., Stucke, VM., Jorritsma, G. and Peter, M. MAP kinase dynamics in responser to pheromones in budding yeast. *Nature cell biology* **12**, 1051-9 (2001).

[35] Kranz, JE., Satterberg, B. and Elion, EA. The MAP kinase Fus3 associates with and phosphorylates the upstream signalling component Ste5. *Genes & development* **8**, 313-327 (1994).

[36] Yu, RC., Pesce, CG., Colman-Lerner, A., Lok, L., Pincus, D., Serra, E., Holl, M., Benjamin, K., Gordon, A. and Brent, R. Fus3 generates negative feedback that improves information transmission in yeast pheromone response. *Nature*, 755-761 (2008).

[37] Brückner, S., Köhler, T., Braus, GH., Heise, B., Bolte, M. and Mösch, HU. Differential regulation of Tec1 by Fus3 and Kss1 confers signaling specificity in yeast pheromone development. *Current genetics* **6**, 331-42 (2004).

[38] Hao, N., Yildirim, N., Nagiec, MJ., Pranell, SC., Errede, B., Dohlman, HG. and Elston, TC. Combined computational and experimental analysis reveals mitogen-activated protein

kinase-mediated feedback phosphorylation as a mechanism for signaling specificity. *Molecular biology of the cell* **16**, 3899-910 (2012).

[39] Maleri, S., Ge, Q., Hackett, E.A., Wang, Q., Dohlman, H.G. and Errede, B. Persistent activation by constitutive Ste7 promotes Kss1-mediated invasive growth but fails to support Fus3-dependent mating in yeast. *Molecular and cellular biology* **24**, 9221-9238 (2004).

[40] Hecker, CM., Jarzombek, J., Wieczorek, J., Karajannis, LS., Koseska, A., Ickstadt, K. and Bastiaens,P. A negative feedback from Fus3 to Ste11 in yeast determines when and how to become a shmoo. unpublished (2014).

[41] Gartner, A., Nasmyth, K and Ammerer, G. Signal transduction in *Saccharomyces cerevisiae* requires tyrosine and threonine phosphorylation of Fus3 and Kss1. *Genes & development* **6**, 1280-1292 (1992).

[42] Bhattacharjya, S., Xu, P., Gingras, R., Shaykhutdinov, R., Wu, C., Whiteway, M. and Ni, F.Solution structure of the dimeric SAM domain of MAPKKK Ste11 and its interaction with the adaptor protein Ste50 from the budding yeast: implicates for Ste11 activation and signal transduction through the Ste50-Ste11 complex. *Journal of molecular biology* **4**, 1071-87 (2004).

[43] Macia, J., Regot, S., Peeters, T., Conde, N., Sole, R. and Posas, F. Dynamic signalling in the Hog1 MAPK pathway relies on high basal signal transduction. *Science signalling* **2**, 1-9 (2009).

[44] Hao, N., Behar, M., Parnell, SC., Torres, MP. And Borchers, CH. A system-biology analysis of feedback inhibition in the Sho1 osmotic-stress-response pathway. *Current biology* **17**, 659-667 (2007).

[45] Schaber, J., Baltanas, R., Bush, A., Klipp, E. and Colman-Lerner, A. Modelling reveals novel role of two parallel signalling pathways and homeostatic feedbacks in yeast. *Molecular systems biology* **8**, article 622 (2012).

[46] Nagiec, MJ. and Dohlman, HG. Checkpoints in a yeast differentiation pathway coordinate signalling during hyperosmotic stress. *PLoS genetics* **1**, Vol. 8 (2012).

[47] Rensing, L. and Ruoff, P. How can yeast cells decide between three activated MAP kinase pathways? A model approach. *Journal of theoretical biology* **257**, 578-587 (2009).

[48] Steffen, M., Petti, A., Aach, J., D´haeseleer, P. and Church, G. Automated modelling of signal transduction networks. *BMC bioinformations* **3**, 34 (2002).

[49] Sabbagah, W., Flatauer, LJ., Bardwell, AJ. And Bardwell,L. Specificity ofMAP kinase signaling in yeast differentiation involves transient versus substained MAPK activation. Molecular cell 3, 683-691 (2001).

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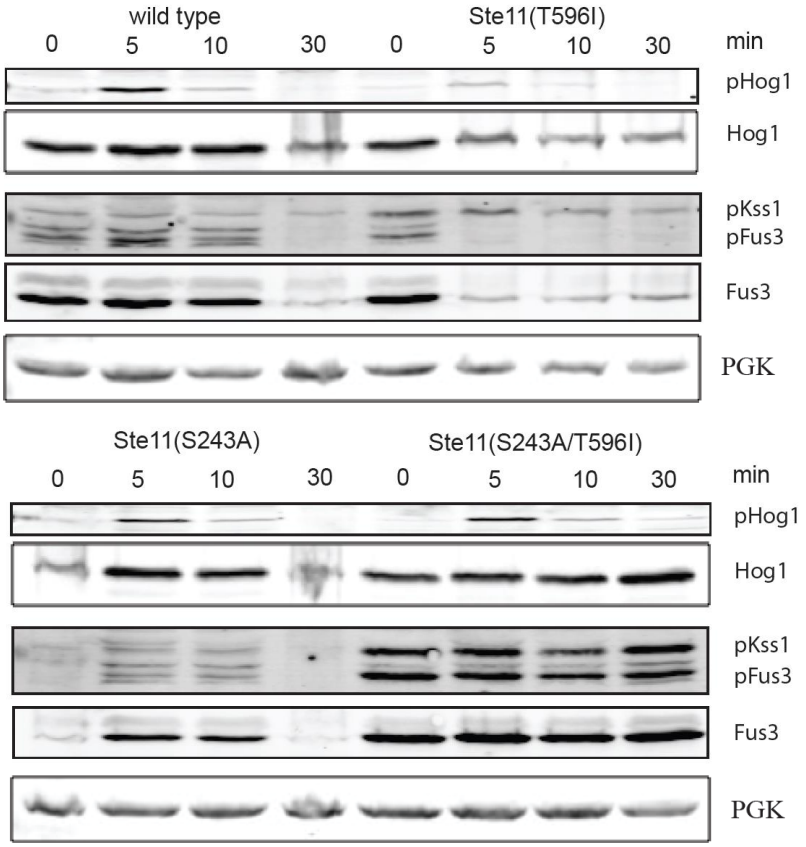
Appendix

Western Blots (stimulated with 0.2 M NaCl) for all investigated strains

Blot 1:



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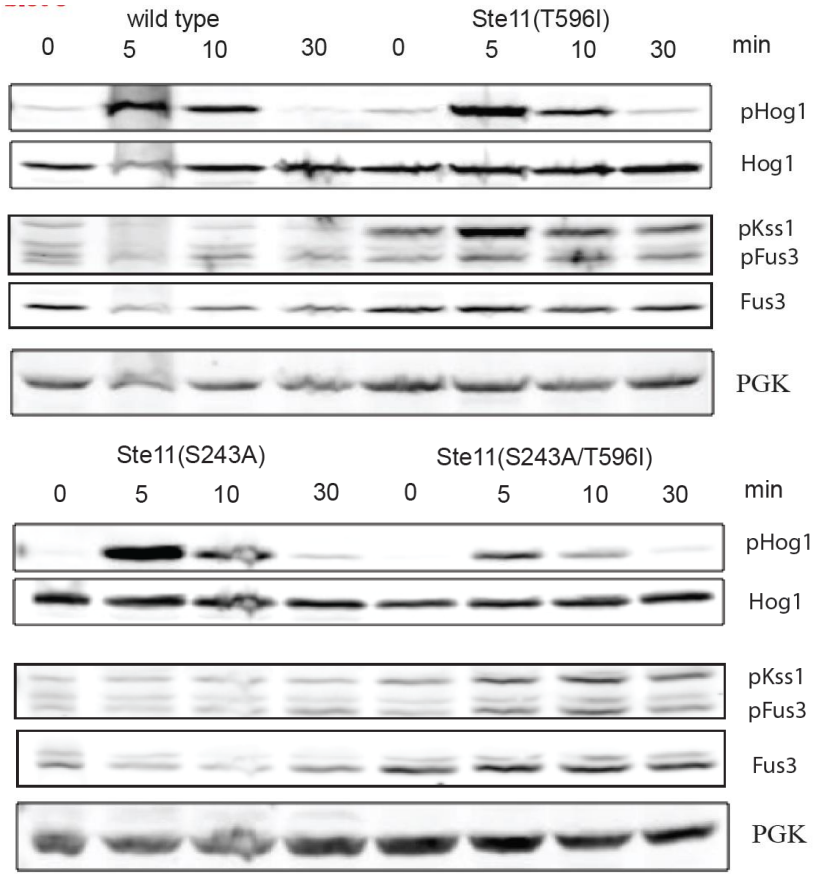

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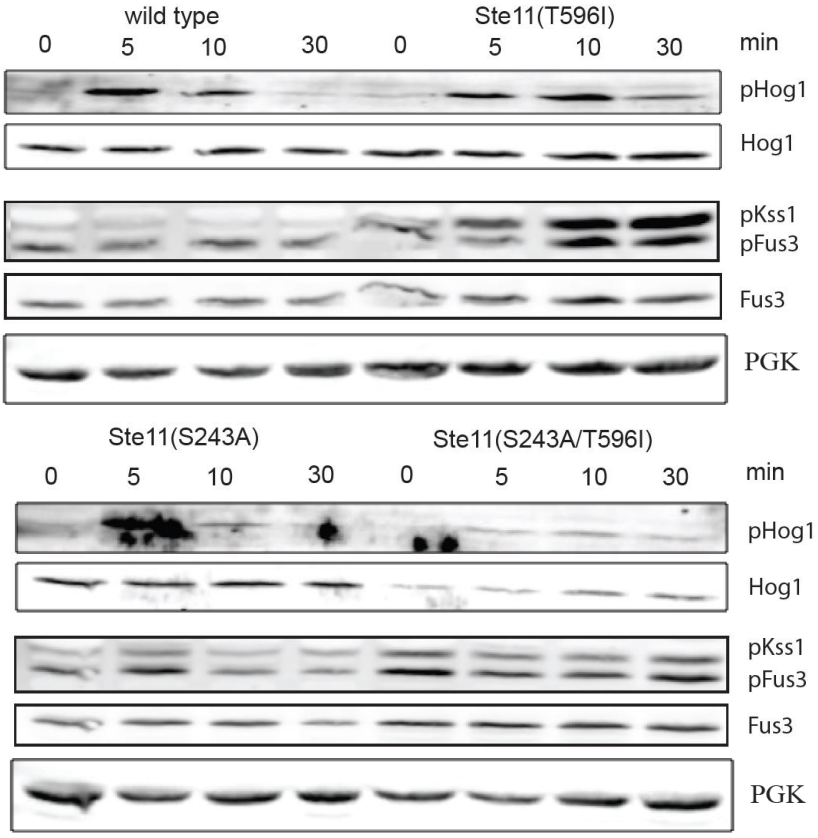
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Hog1
pKss1
pFus3
Fus3
PGK
Ste11(S243A)
Ste11(S243A/T596I)
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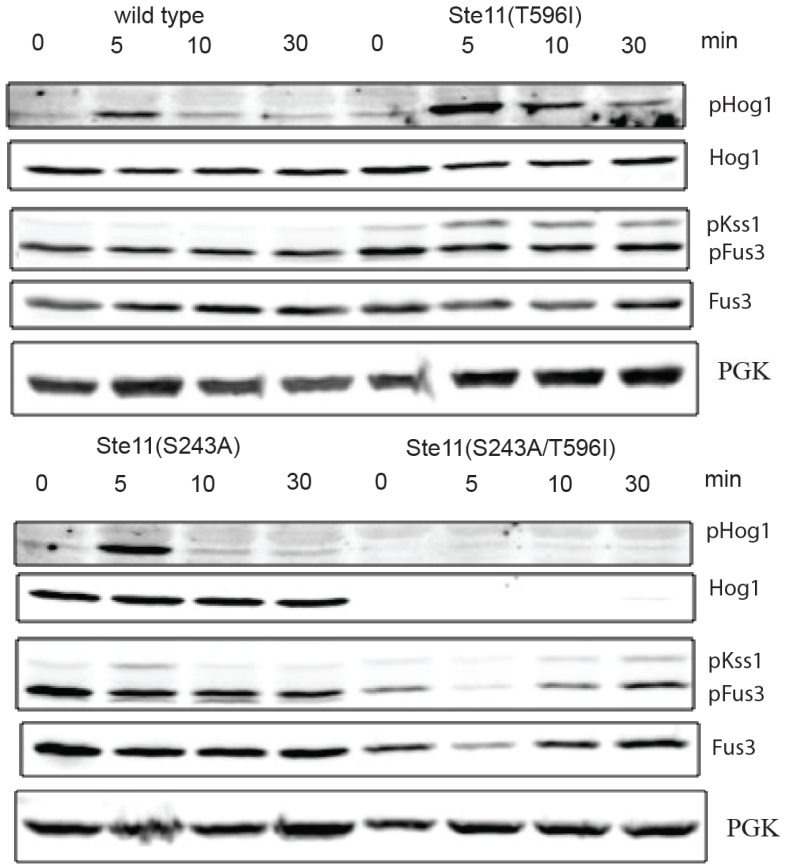
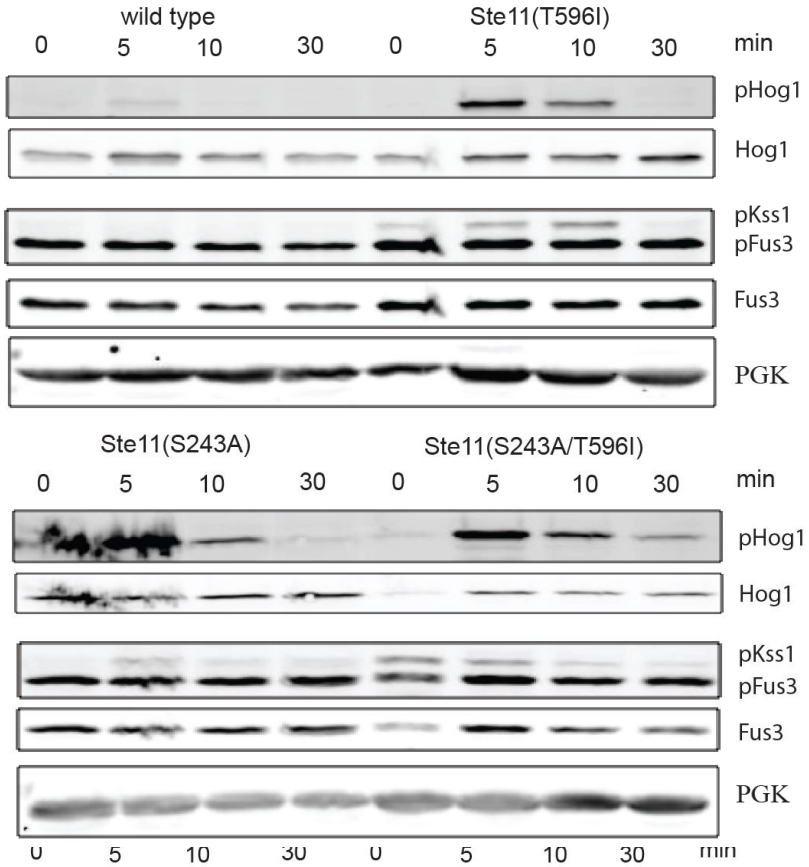
pHog1
Hog1
pKss1
pFus3
Fus3
PGK

Blot 4:


wild type
Ste11(T596I)
0 5 10 30 0 5 10 30 min
pHog1
Hog1
pKss1
pFus3
Fus3
PGK
Ste11(S243A)
Ste11(S243A/T596I)
0 5 10 30 0 5 10 30 min
pHog1
Hog1
pKss1
pFus3
Fus3
PGK

Blot 6: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Hog1
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pFus3
Fus3
PGK
Ste11(S243A)
Ste11(S243A/T596I)
0 5 10 30 0 5 10 30 min
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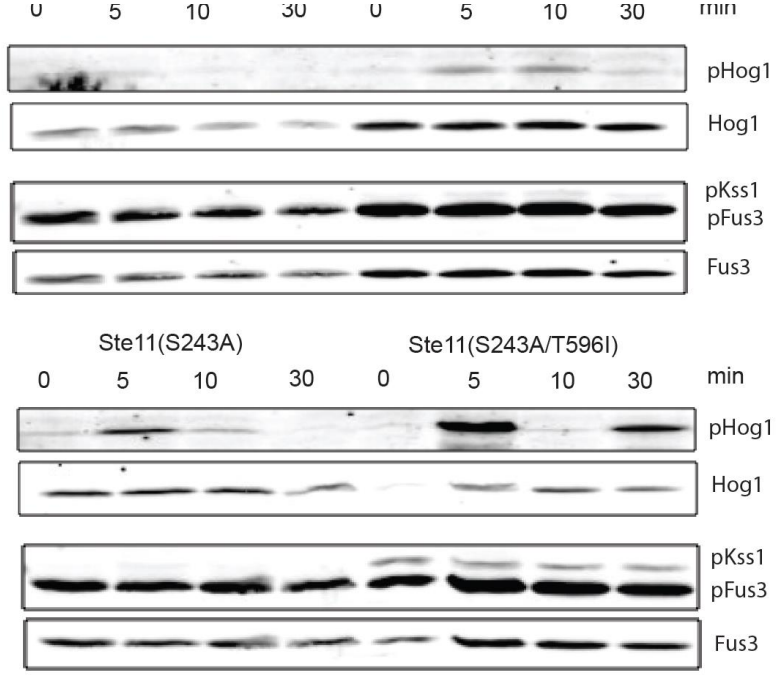
Hog1
pKss1
pFus3
Fus3
PGK

Blot 7: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pKss1
pFus3
Fus3
Ste11(S243A)
Ste11(S243A/T596I)
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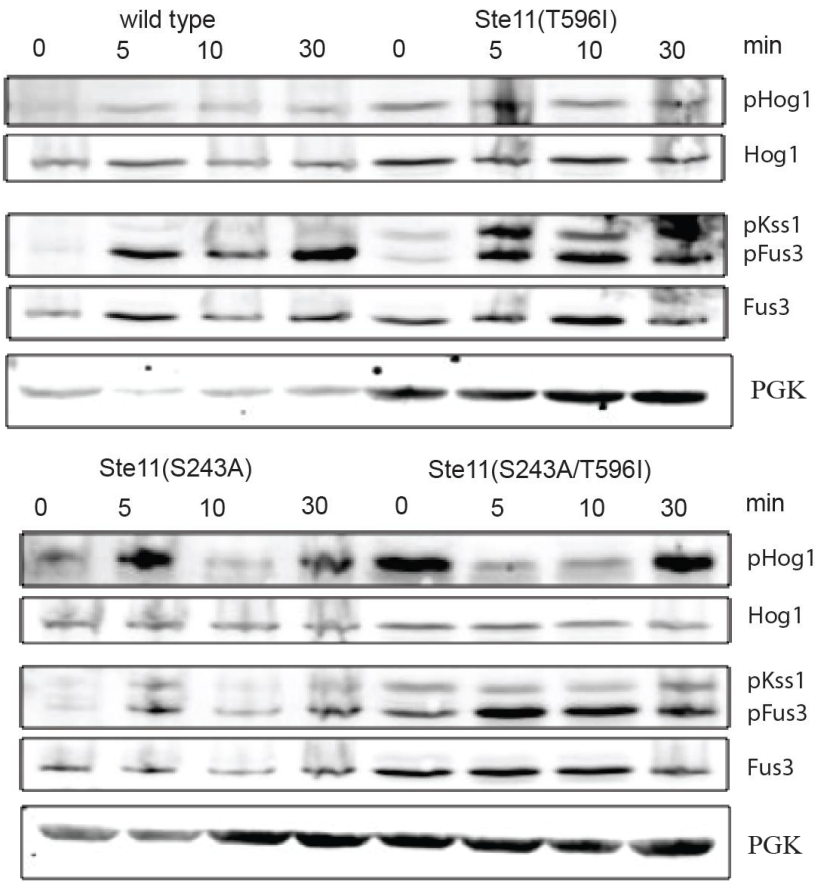
Hog1
pKss1
pFus3
Fus3

Western Blots (stimulated with 100 nM pheromone) for all investigated strains

Blot 1:



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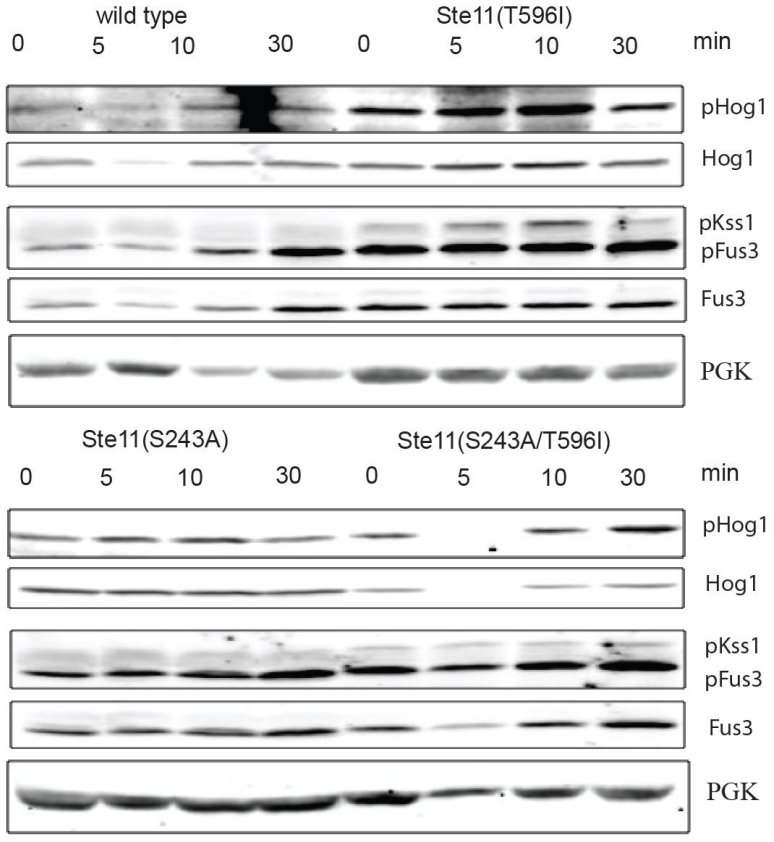

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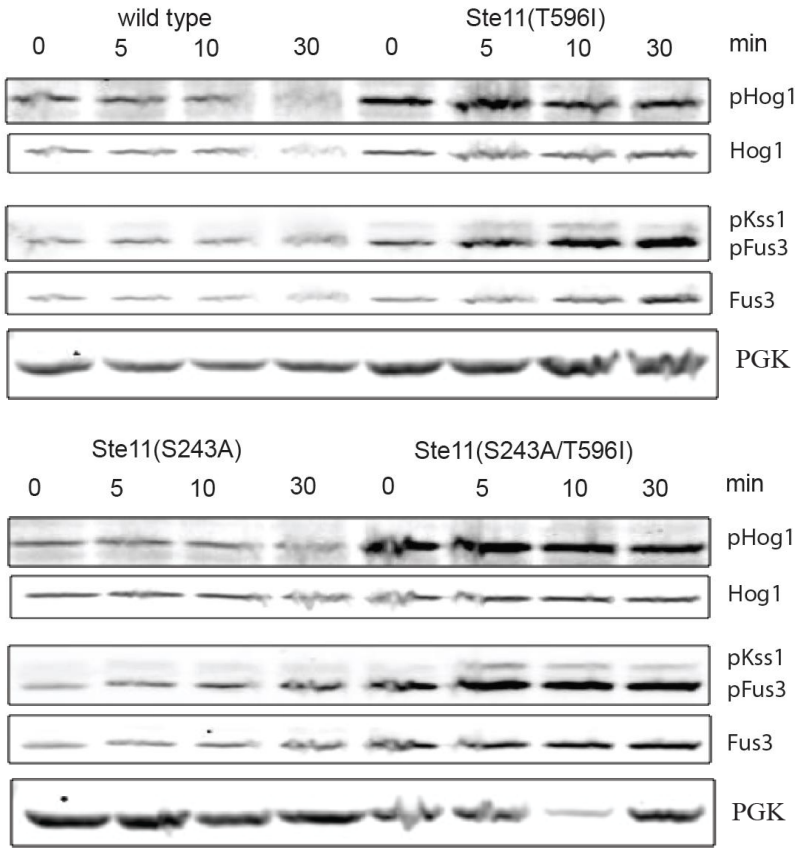
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pHog1
Hog1
pKss1
pFus3
Fus3
PGK
Ste11(S243A)
Ste11(S243A/T596I)
0 5 10 30 0 5 10 30 min
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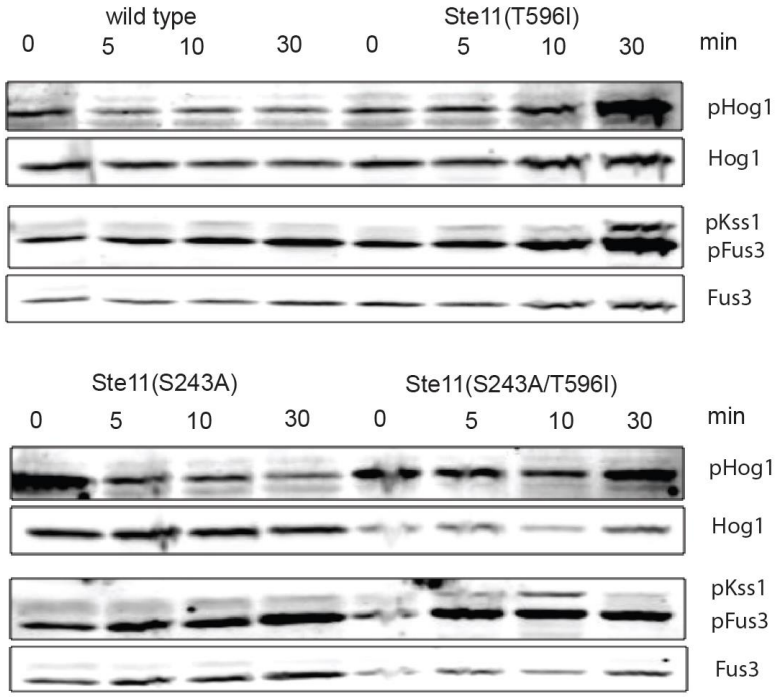
Hog1
pKss1
pFus3
Fus3
PGK

Blot 4: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Hog1
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pFus3
Fus3
Ste11(S243A)
Ste11(S243A/T596I)
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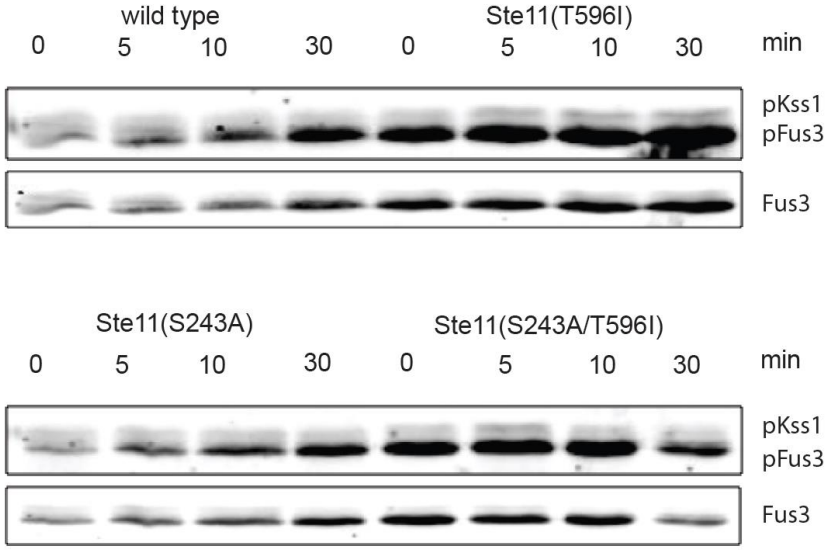
Hog1
pKss1
pFus3
Fus3

Blot 5: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Ste11(S243A/T596I)
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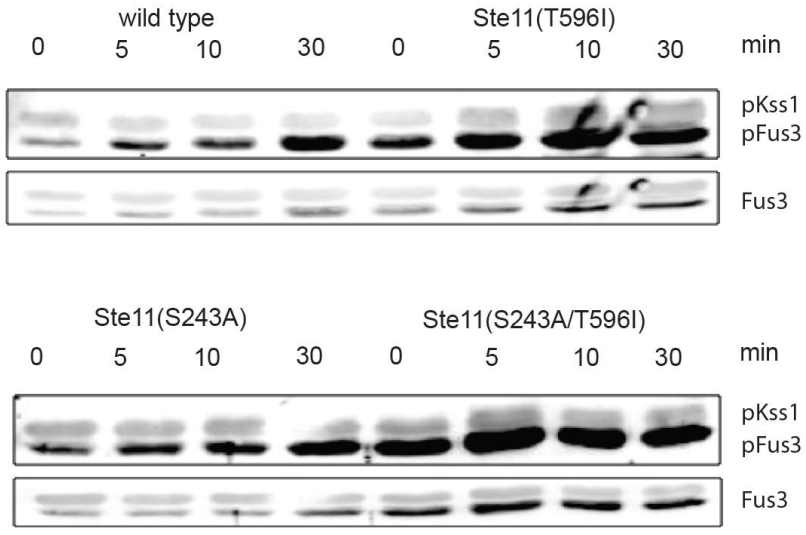
pFus3
Fus3

Blot 6: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Fus3
Ste11(S243A)
Ste11(S243A/T596I)
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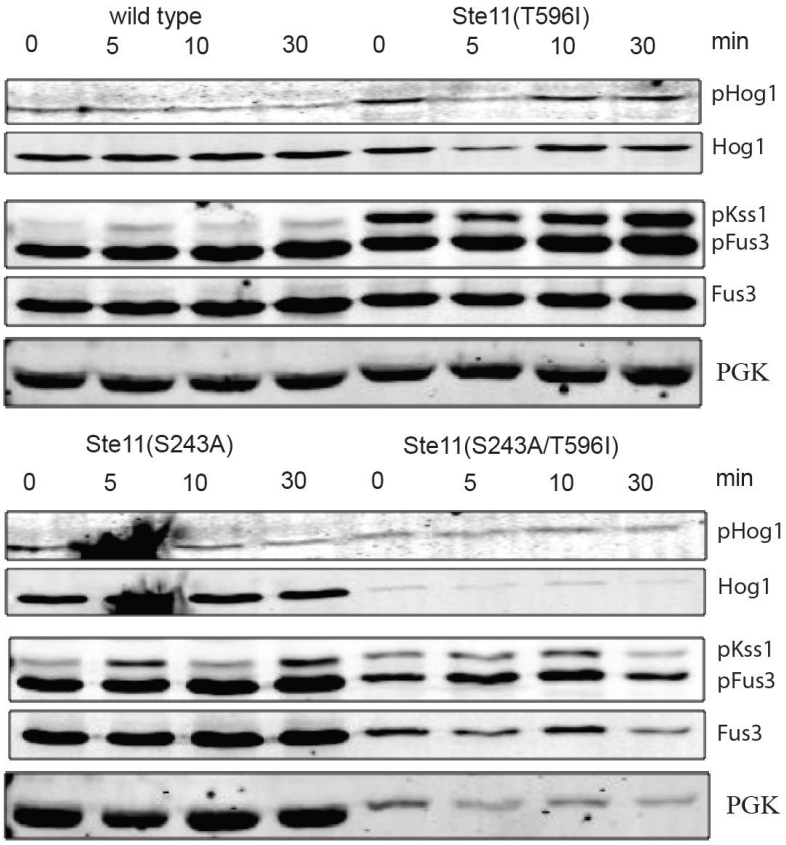
pKss1
pFus3
Fus3

Blot 7: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Hog1
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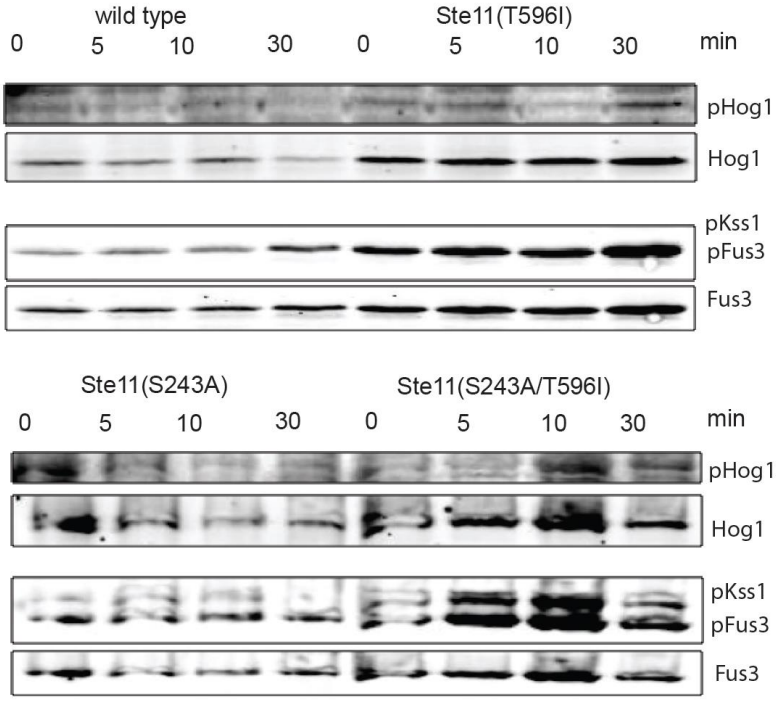
pFus3
Fus3
PGK
Ste11(S243A)
Ste11(S243A/T596I)
0 5 10 30 0 5 10 30 min
pHog1
Hog1
pKss1
pFus3
Fus3
PGK

Blot 8:


wild type
Ste11(T596I)
0 5 10 30 0 5 10 30 min
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Hog1
pKss1
pFus3
Fus3
Ste11(S243A)
Ste11(S243A/T596I)
0 5 10 30 0 5 10 30 min
pHog1
Hog1
pKss1
pFus3
Fus3

Analysis of the Western Blots (stimulated with 0.2 M NaCl)

Hog1/p38MA	0	5	10	30	[min]	arithmetic m	0	5	10	30	[min]
WT	0,008	0,030	0,008	0,007	blot 1	WT	0,038	0,107	0,064	0,028	
CA	0,007	0,048	0,019	0,007		CA	0,044	0,197	0,115	0,046	
SA	0,016	0,047	0,013	0,007		SA	0,034	0,092	0,062	0,043	
DM	0,008	0,114	0,026	0,010		DM	0,358	0,671	0,341	0,254	
WT	0,042	0,311	0,168	0,025	blot 2	standarddivi	0	5	10	30	[min]
CA	0,040	0,358	0,119	0,029		WT	0,027	0,118	0,066	0,025	
SA	0,021	0,336	0,126	0,029		CA	0,049	0,182	0,106	0,043	
DM	0,020	0,111	0,058	0,022		SA	0,019	0,053	0,071	0,031	
						DM	0,360	0,518	0,377	0,233	
WT	0,071	0,152	0,054	0,023	blot 3						
CA	0,023	0,068	0,090	0,033		standarderro	0	5	10	30	[min]
SA	0,056	0,164	0,039	0,060		WT	0,012	0,053	0,029	0,011	
DM	0,067	0,049	0,040	0,034		CA	0,020	0,074	0,043	0,018	
						SA	0,010	0,026	0,032	0,014	
WT	0,055	0,129	0,078	0,070	blot 4	DM	0,180	0,259	0,188	0,117	
CA	0,055	0,235	0,144	0,088							
SA	0,044	0,099	0,051	0,053		(values not calculated)					
DM	0,777	0,989	0,776	0,569							
WT	0,151	0,100	0,113	0,129	blot 6						
CA	0,136	0,454	0,303	0,111							
SA	0,093	0,638	0,187	0,079							
DM	0,532	1,217	0,536	0,251							
WT	0,014	0,018	0,010	0,014	blot 7						
CA	0,005	0,016	0,016	0,009							
SA	0,019	0,058	0,022	0,014							
DM	0,114	0,362	0,025	0,185							

Basal phosphorylation level of Hog1 (0 min)							
	arithmetic m	blot 1	blot 2	blot 3	blot 4	blot 6	blot 7
WT	0,046	0,008	0,042	0,071	0,055	0,151	0,014
CA	0,052	0,007	0,040	0,023	0,055	0,136	0,005
SA	0,053	0,016	0,021	0,056	0,044	0,093	0,019
DM	0,474	0,008	0,020	0,067	0,777	0,532	0,114

standarddivi	standarderror
0,024	0,012
0,051	0,023
0,031	0,015
0,335	0,194

Fus3/AF1018	0	5	10	30	[min]	arithmetic m	0	5	10	30	[min]
WT	0,105	0,104	0,111	0,214	blot 1	WT	0,261	0,243	0,218	0,244	
CA	0,066	0,165	0,123	0,129		CA	0,249	0,270	0,301	0,287	
SA	0,590	0,094	0,099	0,211		SA	0,268	0,242	0,210	0,237	
DM	0,095	0,065	0,065	0,076		DM	0,178	0,122	0,152	0,182	
WT	0,051	0,078	0,062	0,051	blot 2	standarddivi	0	5	10	30	[min]
CA	0,030	0,039	0,065	0,042		WT	0,252	0,199	0,169	0,181	
SA	0,036	0,044	0,062	0,049		CA	0,326	0,295	0,300	0,287	
DM	0,022	0,035	0,043	0,033		SA	0,253	0,220	0,193	0,151	
						DM	0,141	0,084	0,126	0,163	
WT	0,277	0,278	0,262	0,221	blot 3						
CA	0,170	0,172	0,285	0,285		standarderro	0	5	10	30	[min]
SA	0,231	0,301	0,191	0,274		WT	0,126	0,100	0,084	0,090	
DM	0,281	0,186	0,181	0,223		CA	0,124	0,148	0,150	0,144	
						SA	0,146	0,110	0,097	0,075	
WT	0,610	0,510	0,436	0,488	blot 4	DM	0,071	0,042	0,063	0,082	
CA	0,729	0,703	0,729	0,690							
SA	0,538	0,527	0,488	0,412		(values not calculated)					
DM	0,313	0,200	0,317	0,394							

pKss1/PGK	0	5	10	30	[min]
WT	0,107	0,164	0,106	0,167	blot 1
CA	0,351	0,706	0,401	0,220	
SA	0,246	0,252	0,231	0,274	
DM	0,844	0,925	0,571	0,938	
WT	0,129	0,140	0,086	0,155	blot 2
CA	0,219	0,586	0,413	0,234	
SA	0,052	0,065	0,056	0,078	
DM	0,068	0,106	0,162	0,149	
WT	0,195	0,278	0,189	0,134	blot 3
CA	0,343	0,528	1,047	1,600	
SA	0,253	0,735	0,350	0,317	
DM	0,768	0,830	0,518	0,498	
WT	0,138	0,086	0,079	0,109	blot 4
CA	0,311	0,581	0,481	0,417	
SA	0,123	0,248	0,105	0,066	
DM	0,139	0,067	0,127	0,237	
WT	0,138	0,112	0,125	0,143	blot 6
CA	0,294	0,350	0,498	0,159	
SA	0,123	0,299	0,185	0,174	
DM	0,789	0,521	0,248	0,203	

arithmetic m	0	5	10	30	[min]
WT	0,125	0,167	0,115	0,141	
CA	0,304	0,550	0,568	0,526	
SA	0,186	0,383	0,218	0,208	
DM	0,635	0,759	0,366	0,469	

standarddivi	0	5	10	30	[min]
WT	0,037	0,081	0,050	0,026	
CA	0,053	0,130	0,271	0,608	
SA	0,073	0,235	0,102	0,112	
DM	0,332	0,211	0,213	0,339	

standarderror				
WT	0,019	0,040	0,025	0,013
CA	0,024	0,058	0,121	0,272
SA	0,036	0,118	0,051	0,056
DM	0,166	0,122	0,107	0,170

(values not calculated)

Analysis of the Western Blots (stimulated with 100 nM pheromone)

Hog1/p38MA	0	5	10	30	[min]
WT	0,013	0,009	0,013	0,012	blot 1
CA	0,011	0,030	0,012	0,023	
SA	0,011	0,012	0,008	0,012	
DM	0,016	0,007	0,010	0,013	
WT	0,012	0,043	0,047	0,008	blot 2
CA	0,01	0,012	0,015	0,008	
SA	0,004	0,005	0,005	0,004	
DM	0,009	0,029	0,012	0,013	
WT	0,028	0,026	0,023	0,028	blot 3
CA	0,024	0,018	0,018	0,018	
SA	0,026	0,021	0,019	0,016	
DM	0,02	0,024	0,025	0,023	
WT	0,023	0,019	0,011	0,013	blot 7
CA	0,054	0,143	0,05	0,078	
SA	0,022	0,018	0,014	0,014	
DM	0,764	0,701	0,582	0,527	
WT	0,142	0,134	0,139	0,205	blot 8
CA	0,072	0,054	0,038	0,041	
SA	0,040	0,063	0,064	0,071	
DM	0,039	0,030	0,029	0,061	
WT	0,026	0,028	0,033	0,031	blot 4
CA	0,026	0,029	0,024	0,040	
SA	0,035	0,021	0,022	0,024	
DM	0,089	0,056	0,101	0,066	

arithmentic	0	5	10	30	[min]
WT	0,021	0,029	0,025	0,018	
CA	0,038	0,055	0,028	0,040	
SA	0,023	0,024	0,022	0,023	
DM	0,049	0,035	0,042	0,041	

standarddivi	0	5	10	30	[min]
WT	0,007	0,010	0,015	0,010	
CA	0,025	0,051	0,015	0,024	
SA	0,014	0,022	0,021	0,024	
DM	0,036	0,014	0,040	0,026	

standarderro	0	5	10	30	[min]
WT	0,003	0,005	0,007	0,005	
CA	0,011	0,023	0,007	0,011	
SA	0,006	0,010	0,009	0,010	
DM	0,021	0,007	0,020	0,013	

(values not calculated)

Fus3/AF1018	0	5	10	30	[min]
WT	0,365	0,632	0,474	0,594	blot 2
CA	0,701	0,776	0,892	0,93	
SA	0,329	0,417	0,355	0,529	
DM	0,777	1,103	0,809	0,717	
WT	0,699	0,795	0,730	0,815	blot 3
CA	0,893	1,288	1,512	1,300	
SA	0,533	0,669	0,715	0,724	
DM	0,697	1,386	1,313	1,094	
WT	0,646	0,832	0,681	0,676	blot6
CA	1,096	1,943	2,083	2,346	
SA	0,599	0,853	1,070	2,240	
DM	2,215	4,165	4,336	4,423	
WT	1,701	1,917	1,815	2,351	blot 7
CA	2,278	3,172	3,189	4,642	
SA	1,810	2,076	1,853	2,679	
DM	2,507	8,306	6,405	5,128	
WT	0,786	1,117	0,979	1,146	blot 8
CA	1,337	1,601	1,570	2,088	
SA	1,085	2,203	2,684	1,711	
DM	1,264	5,058	3,450	3,039	
WT	0,258	0,366	0,410	0,460	blot 5
CA	0,483	0,629	0,618	0,777	
SA	0,312	0,306	0,372	0,444	
DM	0,473	0,674	0,663	0,529	
WT	0,363	0,458	0,577	0,731	blot 4
CA	0,481	0,656	0,722	1,469	
SA	0,404	0,513	0,588	0,746	
DM	0,447	1,181	1,690	0,920	

arithmetic m	0	5	10	30	[min]
WT	0,688	0,874	0,809	0,968	
CA	1,038	1,438	1,417	2,129	
SA	0,725	1,005	1,091	1,296	
DM	1,492	4,004	3,263	2,880	

standarddivi	0	5	10	30	[min]
WT	0,489	0,524	0,481	0,646	
CA	0,630	0,914	0,956	1,336	
SA	0,288	0,795	0,873	0,906	
DM	0,829	2,954	2,285	1,958	

standarderrc	0	5	10	30	[min]
WT	0,185	0,198	0,182	0,244	
CA	0,238	0,346	0,390	0,545	
SA	0,109	0,301	0,330	0,342	
DM	0,371	1,321	1,022	0,875	

(values not calculated)

basal phosphorylation level of Fus3 (0min)

	arithmetic m	blot 3	blot 4	blot 5	blot 6	blot 7	blot 8	blot 9
WT	0,688	0,365	0,699	0,363	0,258	0,646	1,701	0,786
CA	1,038	0,701	0,893	0,481	0,483	1,096	2,278	1,337
SA	0,725	0,329	0,533	0,404	0,312	0,599	1,81	1,085
DM	1,492	0,777	0,697	0,447	0,473	2,215	2,507	1,264

standarddivi	standarderror
0,489	0,185
0,630	0,238
0,546	0,206
0,829	0,414

pKss1/PGK	0	5	10	30	[min]
WT	0,048	0,074	0,290	0,267	blot 1
CA	0,085	0,724	0,380	0,712	
SA	0,070	0,206	0,074	0,215	
DM	0,156	0,253	0,292	0,228	
WT	0,079	0,082	0,182	0,132	blot 2
CA	0,083	0,138	0,174	0,123	
SA	0,039	0,046	0,042	0,036	
DM	0,045	0,092	0,079	0,102	
WT	0,059	0,105	0,104	0,134	blot 3
CA	0,091	0,146	0,147	0,090	
SA	0,065	0,089	0,088	0,077	
DM	0,084	0,200	0,151	0,151	
WT	0,210	0,180	0,142	0,194	blot 7
CA	0,839	0,325	0,549	0,863	
SA	0,142	0,283	0,170	0,288	
DM	0,096	0,114	0,192	0,067	
WT	0,077	0,097	0,102	0,114	blot 4
CA	0,075	0,130	0,109	0,388	
SA	0,201	0,159	0,161	0,119	
DM	0,111	0,219	0,801	0,114	

arithmeticm	0	5	10	30	[min]
WT	0,066	0,107	0,164	0,169	
CA	0,084	0,292	0,272	0,366	
SA	0,103	0,157	0,107	0,147	
DM	0,098	0,175	0,178	0,132	

standarddivi	0	5	10	30	[min]
WT	0,015	0,042	0,078	0,063	
CA	0,006	0,254	0,187	0,357	
SA	0,067	0,094	0,056	0,103	
DM	0,041	0,069	0,089	0,061	

standarderrc	0	5	10	30	[min]
WT	0,007	0,019	0,035	0,028	
CA	0,003	0,114	0,084	0,178	
SA	0,030	0,042	0,025	0,046	
DM	0,018	0,031	0,044	0,027	

(values not calculated)

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