Regulated nuclear import of the hsp70 Ssa4p upon ethanol stress in the budding yeast *Saccharomyces cerevisiae*

By

Rui Zhang

Department of Physiology, McGill University,

Montreal, Quebec, Canada

February, 2006

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Your file Votre référence
ISBN: 978-0-494-32644-2
Our file Notre référence
ISBN: 978-0-494-32644-2

TABLE OF CONTENTS

Abstract

The N-terminal 236 residues of Ssa4p, a hsp70 in budding yeast, are sufficient to target GFP to nuclei in ethanol-treated cells; this transport is mediated by the karyopherin-β Nmd5p. Ssa4p(1-236)-GFP nuclear accumulation upon ethanol exposure depends on the cell surface sensors Wsc1p and Mid2p as well as protein kinase C, an activator of the cell integrity MAPK cascade. I have analyzed the distribution of Ssa4p(1-236)-GFP and Nmd5p-His6-HA in deletion mutants of the cell integrity MAPK cascade and demonstrate that this pathway controls ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP. Protein phosphorylation regulates protein trafficking, and I have studied this modification for Ssa4p(1-236)-GFP, Nmd5p and the nucleoporin Nsp1p. My results suggest that the phosphorylation status of Nmd5p may change when cells are treated with ethanol, and threonine was identified as the putative target residue(s) for modification. Furthermore, Snf1p kinase is required for stress-induced nuclear accumulation of Ssa4p(1-236)-GFP; my data are in line with the idea that Snf1p kinase phosphorylates a threonine residue present at a consensus Snf1p site in Nmd5p. In summary, my studies link the stress-induced nuclear accumulation of Ssa4p(1-236)-GFP to the cell integrity MAPK pathway and Snf1p kinase.

Résumé

Les 236 résidus de la partie N-terminale de Ssa4p, une hsp70 des levures bourgeonnantes, sont suffisant pour cibler la GFP au noyau des cellules traitées à l'éthanol; ce transport étant médié par la karyophérine-β Nmd5p. L'accumulation nucléaire de Ssa4p(1-236)-GFP lors de l'exposition des cellules à l'éthanol dépend des capteurs Wsc1p et Mid2p qui se trouvent à la surface cellulaire, ainsi que de la protéine kinase C, l'activatrice de la cascade contrôlant l'intégrité cellulaire MAPK. J'ai analysé la distribution de Ssa4p(1-236)-GFP et de Nmd5p-His6-HA chez des mutants ne possédant plus la cascade du contrôle de l'intégrité cellulaire MAPK et j'ai ainsi démontré que cette voie contrôle l'accumulation nucléaire de Ssa4p(1-236)-GFP suite à un traitement à l'éthanol. La phosphorylation des protéines régule leurs déplacements, j'ai ainsi étudié cette modification pour Ssa4p(1-236)-GFP, Nmd5p et la nucléoprotéine Nsp1p. Mes résultats suggèrent que l'état de phosphorylation de Nmd5p peut changer lorsque les cellules sont traitées à l'éthanol, et la thréonine fut identifiée comme étant le résidu putatif cible de la phosphorylation. De plus, la kinase Snf1p est nécessaire lors de l'accumulation nucléaire de Ssa4p(1-236)-GFP induite par un stress; mes résultats concordent avec l'idée que la kinase Snf1p phosphoryle un résidu thréonine de Nmd5p consensus à un site de Snf1p. En résumé, mes recherches relient l'accumulation nucléaire de Ssa4p(1-236)-GFP suite à un stress, à la voie

de l'intégrité cellulaire MAPK et à la kinase Snf1p.

Acknowledgements

I would like to express my sincere appreciation to my supervisor, Dr. Ursula Stochaj for giving me the opportunity to conduct such an interesting research project and greatly supporting my graduate studies in scientific guidance, financial aid and editorial assistance. During my research progress, she has been here all the time to discuss every detail of the project with me, answer each question of mine, help me conquer all the technique problems, correct mistakes in the slides for each of my presentation, and help revise my thesis for so many times. She is one of the best supervisors I have ever seen. What I have learned from her is not only the research skills, but also meticulous thinking, persevering pursuing and being extremely responsible for the work.

I would like to sincerely thank my graduate committee members: Dr. John Orlowski, Dr. Howard Bussey and Dr. Peter Swain, for their excellent academic advice on my project.

Warmest thanks go to my colleagues (Xinxin Quan, Mohamed Kodiha and Piotr Banski) in the Stochaj lab for providing me so many helpful suggestions during my research process and such a delighting working environment. Special thanks to Piotr for translating the abstract from English to French. I also would like to sincerely thank our technician Neola Matusiewicz for technical help, encouragement and helping revise my thesis.

I would like to acknowledge Drs. H. Bussey, M. Whiteway, V. Doye, G. Schlenstedt, and M. Johnston for kindly providing us the yeast strains used in this thesis.

Special gratitude goes to Christine Pamplin, Dr. John Orlowski, Dr. Kathleen Cullen and Dr. James Nemes. Without their help and support, I could not go through all the difficulties and complete my graduate studies in McGill University.

A special note of thanks to my friends Naomi Burton-Macleod who helped me on revising my thesis beyond her busy schedule, and Qingwei Deng who has been always there listening to my problems and encouraging me.

At the end, I would like to express the deepest gratitude to my mother Yanling, my father Shiying, my sisters Yi and Ying, and my fiancé Ling. They have given me unconditional love and unwavering support. I would never have done this work without them. Having them as my family is the greatest blessing to me.

Abbreviations

ADP	Adenosine diphosphate
AhR	Aryl hydrocarbon receptor
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cNLS	Classical nuclear localization signal
DAPI	4',6-diamidino-2-phenylindole
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ETOH	Ethanol
FG	Phenylalanine-glycine
FITC	Fluoresecein isothiocyanate
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
Gsp1p	Ran homologue in *Saccharomyces cerevisiae*
GST	Glutathione S-transferase

GTP	Guanosine triphosphate
HA	Hemagglutinin
His	Histidine
HRP	Horseradish peroxidase
Hsc70	Heat shock cognate 70 kDa
HSE	Heat shock element
HSF	Heat shock factor
Hsp	Heat shock protein
Hsp70	Heat shock proetin 70 kDa
Hxk2p	Hexokinase PII
IMAC	Immobilized metal affinity chromatography
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MDa	Megadalton
MST	Mammalian STE20-like kinase
mSTI1	Murine stress-inducible protein 1
NE	Nuclear envelope
NES	Nuclear export signal
NLS	Nuclear localization sequence

NMD5	Nonsense-mediated mRNA decay 5
NPC	Nuclear pore complex
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PKC	Protein kinase C
PMSF	Phenylmethylsulphonylfluoride
PRAK	p38 regulated/activated protein kinase
Prp20p	RCC1 homologue in *Saccharomyces cerevisiae*
Ran	Ras-related nuclear protein
RanBP	Ran-binding protein
RanGAP	RanGTPase activating protein
RanGDP	GDP-bound form of Ran
RanGTP	GTP-bound form of Ran
RCCl	Regulator of chromosome condensation 1
Rna1p	RanGAP1 homologue in *Saccharomyces cerevisiae*
SC	Synthetic complete
SDS	Sodium dodecyl sulfate
Snf1p	Sucrose non-fermenting-1 protein
Srp1p	Karyopherin-α homologue in *Saccharomyces cerevisiae*

SV40 T-Ag	Simian virus T-antigen
TBS	Tris-buffered saline

Contributions of authors

The experiments carried out for Fig. 3-4 of this thesis were designed by Dr. Ursula Stochaj and performed by Rui Zhang. The quantification of Fig. 4A was completed by Rui Zhang and Xinxin Quan. Cell images in Fig. 3 were taken by Dr. Ursula Stochaj and Rui Zhang.

The experiments for Fig. 5-6 of this thesis were devised by Dr. Ursula Stochaj and conducted by Rui Zhang. The plasmid encoding Nmd5p-His6-HA was constructed by Rui Zhang. Cell images in Fig. 5 were taken by Dr. Ursula Stochaj and Rui Zhang.

The experiments carried out for Fig. 7 of this thesis were designed by Dr. Ursula Stochaj. The experiments carried out for Fig. 7A, B, C-a and C-c were performed by Rui Zhang. Optical density readings in Fig. 7B were collected and calculated by Rui Zhang. The experiments for Fig. 7C-b were conduçted by Dr. Ursula Stochaj and Rui Zhang.

The mutant yeast strains used for Fig. 3-7A were kindly provided by Dr. H. Bussey (Montreal, Canada).

The experiments for Fig. 9 of this thesis were devised by Dr. Ursula Stochaj and Rui Zhang, performed by Rui Zhang. Cell images in Fig. 9 were taken by Dr. Ursula Stochaj and Rui Zhang. The yeast strain containing the plasmid encoding GST-Snf1p was kindly provided by Dr. M. Whiteway. The *snf1*Δ yeast strain was generously provided by Dr. M. Johnston (Stanford, USA).

Chapter I-

Literature review

1.1 Nucleocytoplasmic trafficking

1.1.1 Nuclear pore complex (NPC)

The nuclear envelope (NE) compartmentalizes eukaryotic cells into cytoplasm and nucleus, and the NE presents a barrier for the movement of macromolecules larger than 40 kDa [1-3]. For instance, this barrier has to be overcome when mRNAs which are transcribed in the nucleus are exported to the cytoplasm for translation. Likewise, transcription factors synthesized in the cytoplasm need to be imported into the nucleus to regulate gene expression. Bidirectional transport between these two compartments occurs through the nuclear pore complex (NPC), a large proteinaceous structure embedded in the nuclear envelope. The general 3D structure of an NPC is shared in all eukaryotes although there are some variations in their molecular masses. For example, the molecular mass of a *Saccharomyces cerevisiae* NPC is ~60 MDa, smaller than that of NPCs of higher eukaryotes [1]. Data from electron-microscopy images, atomic-force microscopy, and cryoelectron tomography have shown that the NPC is a highly symmetric structure composed of three parts. The central part is a cylindrical ring with eight spokes embedded in the nuclear envelope. On the cytoplasmic side, protein filaments extend from the central ring and spread outwards; in the nucleus, protein filaments are connected with the central ring, but joined together and forming a basket-like structure, as shown in Fig. 1 [1-11]. The yeast NPC consists of about

30 different proteins, termed nucleoporins. In yeast and mammalian cells, approximately one third of the nucleoporins contain phenylalanine-glycine (FG) repeat motifs which play an important role in the translocation of macromolecules through NPCs [5, 9].

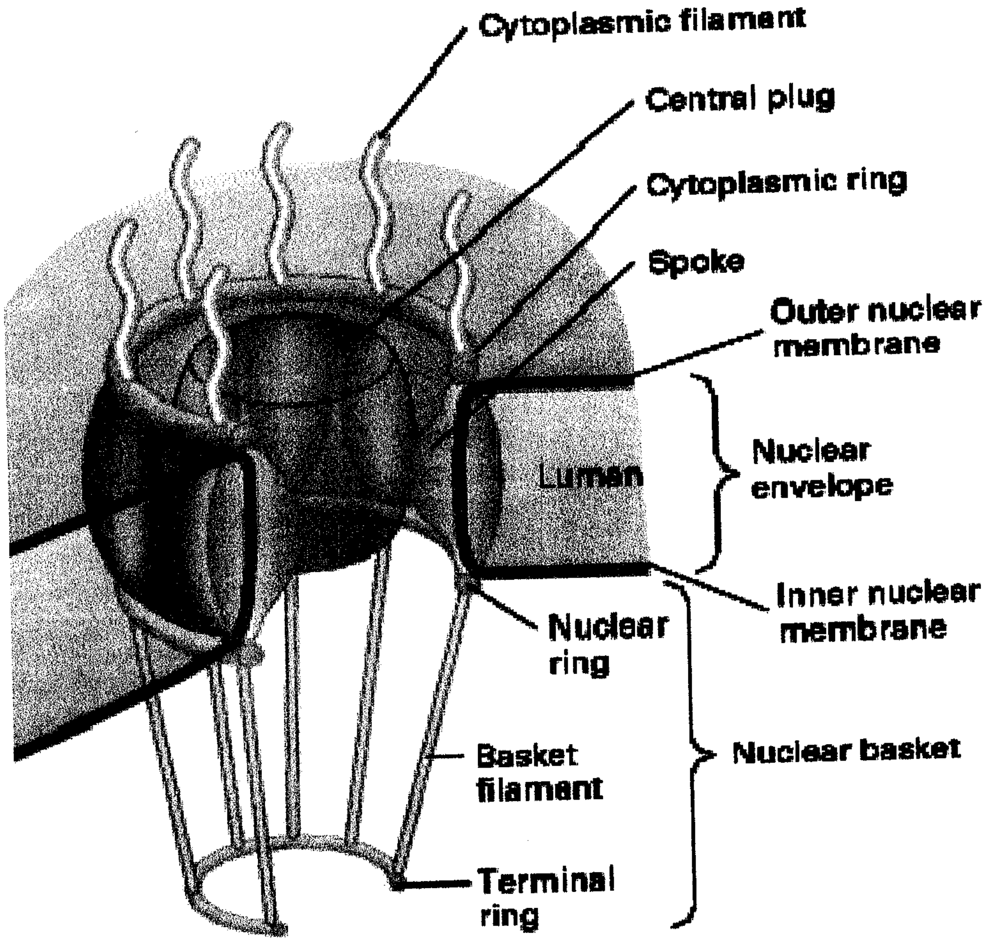


Fig. 1 Simplified model of an NPC (from reference [8])

1.1.2 Nucleocytoplasmic transport of proteins

Generally, there are two mechanisms to transport proteins between nucleus and cytoplasm. Small proteins (less than 40 kDa in molecular mass) can move freely through NPCs by passive diffusion [4, 12, 13], whereas larger proteins move through NPCs by a signal-mediated mechanism. Proteins transported by this mechanism must carry a specific nuclear transport signal which can be recognized and bound by transport receptors or adaptor proteins to form a stable transport complex. This transport pathway requires energy as well as the interaction of the transport apparatus with nucleoporins [1, 9, 13, 14].

There are two classes of nuclear transport signals: nuclear localization sequences (NLSs) and nuclear export signals (NESs). NLSs are carried by cargo proteins which need to be imported into the nucleus. They are recognized by import receptors (also called importins). On the other hand, NESs are carried by cargo proteins which need to be exported from the nucleus to cytoplasm. These cargo proteins are recognized by export receptors (also called exportins). Many NLSs and NESs are short stretches of amino acid residues. The best studied NLSs are monopartite NLSs, such as the signal present in SV40 large-T antigen, and bipartite NLSs, like the NLS of nucleoplasmin. These two classes of NLSs are called classical nuclear localization signals (cNLSs) and the transport pathway of cargo containing a cNLS is called classical nuclear import [15-18].

Nucleocytoplasmic transport requires the interaction between transport receptors, their cargos and NPCs. The direction of nuclear transport depends on the small GTPase Ran/Gsp1p. As shown in Fig. 2 [19], the NLS of a cargo protein is recognized in the cytoplasm and bound directly by one of the karyopherins or through an adaptor protein. The karyopherin/cargo complex then docks at the NPC and translocates through the NPC. Due to the nuclear localization of the Ran/Gsp1p guanine nucleotide exchange factor (RCC1/Prp20p), the small GTPase Ran/Gsp1p in the nucleus is predominantly in a GTP-bound form. When the import complex enters the nucleus, the high concentration of RanGTP/Gsp1pGTP stimulates the dissociation of the import complex and RanGTP/Gsp1pGTP subsequently binds the importin with higher affinity. This leads to the release of cargo proteins in the nucleus and the importin–RanGTP/Gsp1pGTP complex moves back to the cytoplasm. In the cytoplasm, Ran/Gsp1p GTPase-activating protein 1 (RanGAP1/Rna1p) activates the GTPase activity of Ran/Gsp1p which generates Ran/Gsp1pGDP. The activity of RanGAP1/Rna1p is controlled by its interaction with Ran/Gsp1p binding protein 1 or 2 (RanBP1 or RanBP2), which are located exclusively in the cytoplasm. The specific distribution of RanBP1 or RanBP2 determines that the predominant form of the small GTPase Ran/Gsp1p in the cytoplasm is RanGDP/Gsp1pGDP.

For the export cycle, one of exportins binds to the NES of a cargo protein and RanGTP/Gsp1pGTP to form a complex in the nucleus. This complex then translocates through the NPC to the cytoplasm, where RanGAP1/Rna1p stimulates GTP hydrolysis. The export complex thereby dissociates and the cargo is released. The empty exportin recycles back into the nucleus and is ready for the next export cycle [6, 8, 12-15, 18-21].

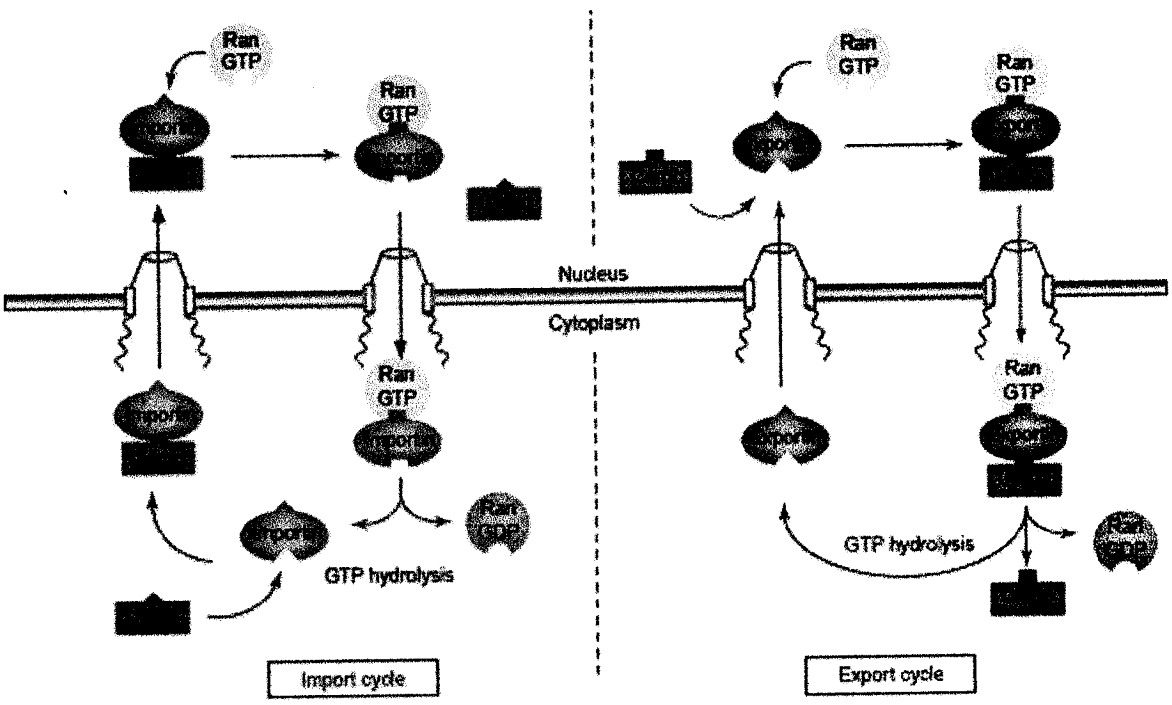


Fig. 2 Nuclear import and export cycles (from reference [19])

1.1.3 Karyopherin-β family

Most of the transport receptors functioning in bidirectional nucleocytoplasmic trafficking are members of the karyopherin-β family. Fourteen members of this family have been identified in the yeast *S. cerevisiae*. More than twenty members are present in mammals [12, 16, 22]. Karyopherin-β proteins are evolutionarily conserved with similar molecular masses (95~145 kDa) although their protein sequence conservation is only ~20%. Atomic structure analyses suggest that each karyopherin-β protein is typically composed of 15-20 HEAT repeats, a helix–loop–helix structure with approximately 40 amino acids [12, 15, 22, 23]. Karyopherin-β proteins contain binding domains for RanGTP/Gsp1pGTP, nucleoporins, cargo or adaptor proteins [12, 15, 22, 23]. So far, structural studies have mainly focused on the mammalian karyopherin-β 1 (also called importin-β1).

Most karyopherin-β proteins function as either import or export receptors. But two of them (yeast Msn5p and mammalian importin 13) participate in both import and export pathways [22, 23]. Since the number of karyopherin-β proteins is smaller than the number of cargos, each karyopherin-β protein is likely to have multiple cargos [22, 23]. Many karyopherin-β proteins bind directly to cargos while some bind to cargo proteins indirectly through an adaptor protein, such as karyopherin-α/Srp1p [12, 15].

1.1.4 Rapid regulation of protein nuclear transport

Regulating the subcellular localization of many proteins is important for cell cycle progression, signaling, development and the response to stress. Protein nuclear transport can be regulated on several levels: by modifying the transport machinery (cargo proteins, transporters or transporter/adaptor complexes), changing the synthesis or degradation of nucleoporins or soluble components of the transport machinery, such as transporters, adaptors or the Ran/Gsp1p system. Numerous experiments have shown that post-translational modification, especially phosphorylation, plays a key role in the rapid regulation of protein nuclear transport [21, 24-26]. Such a modification is most commonly used for cargo proteins where the target residue(s) for phosphorylation are within or adjacent to NLSs or NESs of cargo proteins.

The first study which elucidated the regulation of nuclear protein import through phosphorylation of an NLS was done by Hubner *et al.* and later confirmed for other cargos [27], such as the NLS of mSTI1 (murine stress-inducible protein 1) [28]. Furthermore, the nuclear import of the AhR (aryl hydrocarbon receptor) is inhibited by phosphorylation of its NLS, whereas phosphorylation of the NES of AhR by p38 MAPK may inhibit its nuclear export [29]. Other examples include the phosphorylation of residue Ser315 in the cNLS of p53, which may regulate the nuclear concentration of p53 [30], and Thr374 in Gli1, which is proximal to the

NLS and involved in Gli1 subcellular localization [31].

Additional studies have shown that phosphorylation sites of cargo proteins which affect their subcellular localization could be far away from NLSs or NESs. For instance, phosphorylation of PRAK (p38 regulated/activated protein kinase) is required for PRAK nuclear export, but the phosphorylated site Thr182 is about 150 residues away from its NES [32]. It is possible that phosphorylation of Thr182 in PRAK induces the intermolecular or intramolecular unmasking of the NES of PRAK [32].

Moreover, there is evidence that phosphorylation of transport receptors or adaptor proteins controls nucleocytoplasmic protein trafficking. For example, Srp1p, the yeast homologue of mammalian karyopherin-α, is phosphorylated upon binding to NLS peptides *in vitro* [33]. In general, phosphorylation could regulate nuclear transport of proteins by changing the conformation of component(s) of the transport apparatus. This could alter the affinity between an NLS or NES and its transport receptor or adaptor protein, thereby changing the efficiency of transport.

Although phosphorylation provides an essential regulation mechanism, other post-translational modifications, like methylation, acetylation, sumoylation, ubiquitination are also involved in trafficking control. For instance, p300-mediated methylation of karyopherin-α1 reduces nuclear import of HuR, a RNA-binding protein [34]. Moreover, ubiquitination of p53, methylation of RNA helicase A and sumoylation of Smad4 also play a role in regulating protein localization [35-37].

In addition to modifications of cargos, transport receptors or adaptor/receptor complexes, the Ran/Gsp1p system and NPCs participate in the control of nucleocytoplasmic trafficking. For example, the ratio of RanGTP:RanGDP is critical in regulating the nuclear level of RanGTP, which determines the direction of the transport [38]. As well, MAPK-mediated phosphorylation of p62, an essential FG-nucleoporin, increases the nuclear import of STAT3 in neurons [39].

1.2 Heat shock protein 70 kDa

1.2.1 Heat shock protein family

Heat shock proteins (hsps) are highly conserved in function between prokaryotic and eukaryotic cells and well known for their primary function as molecular chaperones. Members of the hsp family are categorized according to their molecular masses, such as hsp40s, hsp70s and vary in molecular mass from 10 kDa to around 150 kDa [40]. Although hsps were initially identified as inducible proteins whose expression is drastically up-regulated in response to elevated temperatures, they include both inducible and constitutively expressed members [41]. Hsps play a broad role in a large number of cellular processes. Under normal growth conditions, they protect nascent immature proteins from degradation and chaperone them to the right subcellular compartments. Hsps are also called "stress proteins" due to their essential roles in cellular protection in response to stresses.

1.2.2 Yeast hsp70s

The budding yeast *S. cerevisiae* has at least fourteen hsp70 members which are grouped into five functionally distinct subfamilies: *SSA, SSB, SSC, SSD* and *SSE* (Stress Seventy subfamilies) [42-44]. *SSC* subfamily members are mitochondrial proteins whereas the *SSD* member Kar2p resides in the endoplasmic reticulum [45-47]. Members of *SSA, SSB, SSE* (mammalian *HSP110* homologues) subfamilies are localized primarily in the cytosol [44, 48, 49].

Although *SSA* and *SSB* subfamilies share 60% identity in their protein sequences, they do have distinct functions. *SSA* family members are located in the cytoplasm and the nucleus, shuttling between the two compartments under normal conditions [50]. The *SSA* subfamily, which contains the four members *SSA1-SSA4*, is essential for growth; at least one *SSA* protein must be synthesized constitutively to keep cells viable. Ssa1p and Ssa2p are constitutively expressed under normal growth conditions, while Ssa3p and Ssa4p are strongly induced upon heat shock [42]. The *SSB* subfamily has two genes encoding Ssb1p and Ssb2p, respectively, which bind directly to newly synthesized polypeptides present in polysomes during translation elongation [43, 48, 51, 52]. Deletion of either *SSB* gene will lead to a cold-sensitive phenotype [51].

1.2.3 Regulation of hsp70 expression

Hsp70 synthesis is induced by activated heat shock factors (HSFs). In mammalian and *Drosophila* cells, HSFs are normally bound by hsps, mostly hsp90, which mask the NLS of HSF in the cytosol. Upon stress, such as heat shock or high concentration [6% (v/v) or higher] of ethanol, hsp90 bind to a bulk of misfolded proteins, which results in the release of HSFs. Released HSFs are then phosphorylated and form homotrimers. They are transported into the nucleus and bind heat shock elements (HSEs) in the promoter region of *HSP70* genes to stimulate transcription. By contrast, in *S. cerevisiae*, HSFs constitutively bind to HSE in the *HSP70* promoter. The activation of yeast HSF is achieved by phosphorylation of HSF on residue Ser460 [40, 52, 53].

1.2.4 Structure of hsp70s

The general structure of hsp70s is highly conserved. All hsp70s contain three functionally important domains: an N-terminal ATPase domain, a peptide-binding domain and a C-terminal EEVD domain. When hsp70 is in the ATP-bound form, the N-terminal ATPase domain binds and hydrolyzes ATP to ADP, which leads to a conformational change in hsp70 and induces tight binding of substrates by the peptide-binding domain. The C-terminal EEVD domain is highly conserved in vertebrates and functions as a “lid”. Substrates have access to the peptide-binding domain only when this “lid” is open. The on-and-off switch of the “lid” is controlled by its interaction with co-chaperones of hsp70, such as hsp40 [43, 52, 54-56].

1.2.5 Functions of hsp70s

Hsp70 proteins function in diverse cellular processes through their major role as molecular chaperones. Constitutively expressed hsp70s bind to newly translated proteins and prevent their misfolding and degradation under normal growth conditions. Upon stress, such as heat, UV light, cytotoxic agents, osmotic changes, high concentration of ethanol and nutrition starvation, more hsp70s are synthesized to help recover misfolded or denatured proteins. Functions of hsp70s range from protein degradation to cellular signaling and apoptosis, which make it a potential target in clinical applications [51, 54, 55, 57-60]. For example, overexpression of hsp70 and its co-chaperones could help decrease protein misfolding and abnormal aggregations in some neurodegenerative diseases, such as Alzheimer’s disease [55].

1.3 Yeast cell wall integrity MAP kinase cascade

The cell wall integrity MAPK pathway is one of five MAPK signaling pathways in the yeast *S. cerevisiae* which also include the mating pathway, the filamentation-invasion pathway, the high-osmolarity growth pathway and the spore wall assembly pathway [61, 62].

The cell wall integrity pathway functions in the cellular response to signals threatening the cell wall integrity, such as budding and environmental cell wall stresses, like heat, osmotic changes and oxidants. The response includes activating the cell wall biosynthetic pathway and regulation of cytoskeleton polarization.

The yeast cell wall integrity pathway is composed of a family of cell surface sensors, including Wsc1p-3p, Mid2p and Mtl1p, the effectors of cell surface sensors, the small GTPase Rho1p and the downstream Pkc1p-MAP kinase cascade [63].

The cell surface sensors perceive cell wall stresses and then induce Rom1/2 GEF (guanine nucleotide exchange factor) to stimulate nucleotide exchange on Rho1p. Activated Rho1p subsequently activates the downstream Pkc1p-MAP kinase cascade and ultimately several cell wall biosynthesis enzymes, such as β-1,3-glucan synthase [61-63] .

The cell wall integrity Pkc1-MAP kinase cascade is comprised of a linear series of protein kinases which activate each other in the order of Pkc1p, a MAP kinase

kinase kinase (Bck1p), a pair of redundant MAP kinase kinases (Mkk1p and Mkk2p), and a MAP kinase (Slt2p, also know as Mpk1p). The first component of the cell integrity Pkc1p-MAPK cascade is Pkc1p, the yeast homologue of mammalian protein kinase C. Without additional osmotic support, *pkc1* mutant cells tend to lyse even under normal conditions, whereas destroying the function of either Bck1p, Slt2p or both Mkk1p and Mkk2p results in lysis only when cells are exposed to cell wall stress, such as heat shock. This indicates that in addition to the cell integrity MAPK cascade other targets exist for Pkc1p [62, 63].

Bck1p, Mkk1p and Mkk2p, mainly localize to the cytoplasm whereas Slt2p mostly localizes to the nucleus under normal growth conditions, but rapidly translocates to the cytoplasm upon cell wall stress [63].

Only a few direct downstream targets of Slt2p have been identified so far. They include Mck1p, a homologue of mammalian glycogen synthase kinase 3, two transcription factors Rlm1p and the SBF complex (Swi4/Swi6) and several protein phosphatases (Ptp2p, Ptp3p, Msg5p, Sdp1p) which reciprocally down-regulate Slt2p [61, 63-66].

1.4 Snf1p kinase

Sucrose non-fermenting 1 (Snf1p) kinase, a yeast serine/threonine protein kinase, is the homologue of mammalian AMP-activated protein kinase (AMPK). Snf1p kinase is an important regulator of carbon source metabolism; it up-regulates the transcription of glucose-repressed genes in response to low concentration of glucose in the growth medium. However, the enzyme also has multiple functions when cells are exposed to various stresses including nutrient deprivation, osmotic stress and heat shock [67-69].

Snf1p kinase is a trimeric complex comprised of three subunits, a catalytic α-subunit (Snf1p), a β-subunit encoded by one of three different genes *SIP1/SIP2/GAL83* and a regulatory γ-subunit encoded by *SNF4* [68, 70]. The subcellular localization of Snf1p kinase is determined by isoforms of the β-subunit [71]. Both Snf1p kinase and AMPK phosphorylate substrates containing a target sequence ΦXRXX*S*XXXΦ, where Φ represents a hydrophobic amino acid residue (M, V, L, I or F) [69, 72-75]. Nevertheless, Snf1p kinase can also phosphorylate residues which are not contained in the above consensus sequence, as exemplified by Ser10 of histone H3 [74, 76].

The regulatory γ-subunit binds to the catalytic α-subunit, which keeps Snf1p dephosphorylated. Thus, the kinase complex is inactive when cells grow in medium with glucose as the main carbon source. When the concentration of glucose is low or any of the negative regulators of Snf1p, such as the glycolytic enzyme hexokinase PII (Hxk2p) or Reg1p [77, 78], are removed, Snf1p is phosphorylated on residue Thr210 [79-81]. This phosphorylation is mediated by one of the upstream kinases Pak1p, Tos3p and Elm1p [81] and results in activation of Snf1p kinase. In the absence of activating stimuli Snf1p is dephosphorylated and thereby inactivated [69].

As discussed above, previous studies have shown that nuclear transport of proteins can be regulated by different mechanisms. Hsp70, as an important cellular chaperone, can be found in different subcellular organelles such as the nucleus. However, the mechanisms that control hsp70s trafficking, are not fully understood. Chapter-II will present my work that focused on the regulation of hsp70 nuclear import in response to ethanol stress, using the budding yeast *S. cerevisiae* as a model system.

Chapter II-

Regulated nuclear import of the hsp70 Ssa4p upon ethanol stress in the budding yeast *Saccharomyces cerevisiae*

2.1 Introduction

After exposure to many environmental stresses, cells initiate a large number of molecular responses and activate signaling pathways. In particular, members of the hsp70/hsc70 family are essential for the survival and recovery from various insults. Hsp70s have a key role as molecular chaperones that promote the recovery of nuclear functions in stressed cells [82-84].The high degree of hsp70/hsc70 conservation among different organisms as well as the conservation of nucleocytoplasmic trafficking in eukaryotic cells makes the budding yeast *Saccharomyces cerevisiae* an ideal model system to study hsp70s nuclear transport.

Like several other hsp70s, the synthesis of the cytoplasmic yeast protein Ssa4p is highly induced upon ethanol stress (above 4-6% v/v) [53]. Ssa4p translocates into nuclei when cells are exposed to 10% ethanol whereas other types of stress, such as osmotic or oxidative stress, fail to trigger the nuclear accumulation of Ssa4p [85]. Interestingly, the N-terminal 236 amino acid residues of Ssa4p, which do not contain a potential classical NLS, are sufficient to target the passenger protein GFP to nuclei in ethanol-treated cells [85]. This suggests that nuclear accumulation of Ssa4p upon ethanol treatment occurs through a non-classical nuclear import pathway. Indeed, previous studies identified Nmd5p, a member of the karyopherin-β family, as the carrier that imports Ssa4p into nuclei of ethanol-stressed cells. This non-classical pathway requires the small GTPase Gsp1p, the GTPase-activating protein Rna1p, and the guanine nucleotide exchange factor Prp20p. However, it does not depend on Srp1p, the yeast homologue of mammalian importin-α, which is a subunit of the classical import receptor [85].

Furthermore, Ssa4p(1-236)-GFP fails to accumulate in nuclei of mutants that are lacking Wsc1p and Mid2p, the major sensors of the cell integrity pathway, or protein kinase C (Pkc1p) [85]. Pkc1p is the only known upstream activator of the cell integrity MAPK cascade so far; however, Pkc1p can also control protein localization independent of the cell integrity pathway [63, 86]. It was therefore important to investigate a possible link between ethanol-induced Ssa4p nuclear accumulation and Pkc1p-MAPK cascade. The cell wall integrity MAPK cascade consists of Bck1p, Mkk1p/Mkk2p and Slt2p. Several downstream targets of Slt2p, such as Mck1p, a homologue of mammalian glycogen synthase kinase 3, transcription factors Rlm1p and the SBF complex (Swi4/Swi6), have been identified [61, 63-66].

Recent studies from our lab have shown that upon 10% ethanol exposure Ssa4p(1-236)-GFP fails to accumulate in nuclei of cells that are lacking a functional Snf1p kinase (unpublished data). Snf1p (Sucrose non-fermenting 1) kinase is a serine/threonine protein kinase that is conserved in fungi, plants and mammals. It participates in the regulation of transcription and also in the cellular response to a variety of environmental stresses, which include nutrient limitation, salt and heat stress [67, 68, 80]. Snf1p recognizes substrates carrying a consensus sequence ΦXRXX*S*XXXΦ , where "Φ" is a hydrophobic residue (M, V, L, I or F) [69, 72-75]. However, Snf1p kinase can also phosphorylate some motifs that do not fit this sequence, for example Ser10 of histone H3 [74, 76].

2.2 Rationale and Objectives

Data from our group show that the N-terminal portion of Ssa4p accumulates in nuclei of ethanol-stressed yeast cells via a non-classical import pathway. This route needs Nmd5p as a carrier, cell surface sensors Wsc1p and Mid2p as well as Pkc1p [85]. My work focused on the molecular mechanism mediating nuclear import of the N-terminal portion of Ssa4p in ethanol-treated *S. cerevisiae*. Since Wsc1p, Mid2p and Pkc1p are components of the cell integrity pathway and Pkc1p is the upstream activator of the cell integrity MAPK cascade, it was possible that the cell integrity Pkc1p-MAPK cascade is involved in the nuclear accumulation of Ssa4p(1-236)-GFP in ethanol-stressed cells. I have tested this hypothesis by localizing Ssa4p(1-236)-GFP as well as Nmd5p-His6-HA upon ethanol exposure in deletion mutants that are missing one of the components of the cell integrity Pkc1p-MAPK pathway.

Post-translational modifications, especially phosphorylation, play an important role in the rapid regulation of nucleocytoplasmic protein trafficking [24, 87, 88]. We hypothesized that phosphorylation of Ssa4p(1-236)-GFP, its transport apparatus or the NPC may contribute to the nuclear accumulation of Ssa4p(1-236)-GFP in response to ethanol exposure. To test this model, I have begun to analyze the post-translational modification of the cargo Ssa4p(1-236)-GFP, its carrier Nmd5p and the essential nucleoporin Nsp1p.

2.3 Materials and Methods

Yeast stains, growth conditions and exposure of stress—Yeast strains used in this study are listed in Table 1. RS453 was used as the wild type strain unless indicated otherwise. Upon transformation, cells were selected in drop-out media. Non-transformed cells were grown in SC-medium or YEPD medium (2% Bacto-peptone, 2% glucose, 1% yeast extract). Expression of genes under the control of a *GALS* or *GAL1* promoter was induced by overnight growth on selective media with 2% galactose at room temperature. The growth media of strains carrying mutations in one of the components of the cell integrity pathway were supplemented with 10% sorbitol. To analyze the effect of ethanol stress, cells were incubated with 10% (v/v) ethanol for 2 or 10 minutes at room temperature as shown in the figures.

Table 1. Yeast strains used in this study

Strain	***Genotype***	***Source***
RS453	MATa *ade2 leu2 ura3 trp1 his3*	V. Doye (Paris, France)
nmd5Δ	MATa *ura3 leu2 his3 nmd5::HIS3*	G. Schlenstedt (Homburg, Germany)
BY4741	MATa *his3 leu2 met15 ura3*	H. Bussey (Montreal, Canada)
BY4743	MATa/α *his3/his3 leu2/leu2 lys2/LYS MET15/met15 ura3/ura3*	H. Bussey
BY4743 *pkc1Δ*	*pkc1:: kanMX4/PKC1*	H. Bussey
BY4741 *bck1Δ*	*bck1:: kanMX4*	H. Bussey
BY4741 *mkk1Δ*	*mkk1:: kanMX4*	H. Bussey
BY4741 *mkk2Δ*	*mkk2::kanMX4*	H. Bussey
BY4743 *slt2Δ*	*slt2:: kanMX4/SLT2*	H. Bussey
BY4741 *mck1Δ*	*mck1:: kanMX4*	H. Bussey
YM3696	MATa *lys2-801:: BM1499 snf1 ura3-52 his3 ade2-101 lys2-801 LEU2:: GAL1-LACZ*	M. Johnston (Stanford, USA) [89]
C6	MATa *leu2 ura3 his3 ade8 trp1 ras1::HIS3 ras2* [YCp*LYS2 RAS1*] [pEG(KG)::*SNF1*]	M. Whiteway (Montreal, Canada) [90, 91]

Plasmid construction and transformation—Plasmids with yeast vectors used in this study are listed in Table 2. To obtain a gene fusion encoding Nmd5p-His6-HA, a forward oligonucleotide (5'-TATGGATCCTACCCTTACGACGTTCCTGATTACGCTGCAAGCTT-3') and a reverse oligonucleotide (5'-ATACCTAGGATGGGAATGCTGCAAGGACTAATGCGACGTTCGAA-3') were annealed and inserted into a plasmid coding for Nmd5p-His6. This generated a fusion of the Nmd5p-His6 coding region followed by a HA-tag. All DNA manipulations were carried out in *E. coli* strain XL1-Blue and verified by sequencing. For expression in yeast cells, the fusion gene Nmd5p-His6-HA was cloned into the centromeric plasmids p2248 with *HIS3* marker, p2247 with *TRP1* marker or p678 with *URA3* marker. In these three plasmids, gene expression is under the control of a *GALS* promoter. To obtain a plasmid encoding Ssa4p(1-236)-GFP with *HIS3* as selectable marker, a fragment containing *GAL1* promoter and the Ssa4p(1-236)-GFP fusion gene was inserted into plasmid p2272 containing the *HIS3* marker. The other plasmids encoding Ssa4p(1-236)-GFP or Nmd5p-His6 were described previously [85].

Competent yeast cells were prepared for transformation following standard procedures [91a]. 50-100 μl of competent yeast cells were resuspended in a mixture of 240 μl 50% (v/v) PEG3350, 36 μl 1 M lithium acetate, 50 μl 1 mg/ml salmon sperm DNA and 10 μl plasmid DNA. The cell suspension was incubated 30 minutes at 30°C and followed by 20 minutes at 42°C. Cells were collected by centrifugation, resuspended in 150 μl distilled H_2O and plated on drop out medium containing 2% glucose.

Table 2. Yeast plasmids used in this study

Plasmid	***Origin of replication***	***Protein encoded***	***Selective marker***	***Promoter***
p929	2μ	Ssa4p(1-236)-GFP	*URA3*	*GAL1*
p2276	CEN	Ssa4p(1-236)-GFP	*HIS3*	*GAL1*
pEG(KG)::*SNF1*	2μ	GST-Snf1p	*URA3, LEU2*	*GAL1*
p2279	CEN	Nmd5p-His6-HA	*HIS3*	*GALS*
p2131	CEN	Nmd5p-His6	*LEU2*	*GALS*
p953	2μ	GFP	*URA3*	*GAL1*
p2248	CEN	Vector	*HIS3*	*GALS*
p2247	2μ	Vector	*TRP1*	*GALS*
p678	CEN	Vector	*URA3*	*GALS*
p2272	CEN	Vector	*HIS3*	—

DNA preparation from yeast cells—Yeast cells carrying a plasmid encoding GST-Snf1p were grown overnight, collected by centrifugation and lysed in 2%

Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA, phenol:chloroform:isoamyl alcohol (25:24:1) by vortexing with glass beads. Plasmid DNA in the aqueous layer was obtained after centrifugation of the cell lysate and purified by ethanol precipitation.

Fluorescence microscopy— All steps were carried out at room temperature. Yeast cells were cultured and exposed to ethanol stress as detailed above. Cells were fixed with 3.7% formaldehyde for 10 minutes, collected by centrifugation, washed twice with P-solution [0.1 M potassium phosphate (pH 6.5) and 20% (w/v) sorbitol] and immobilized on polylysine-coated 15-well slides. Samples were dehydrated in ice-cold methanol for 4 minutes and ice-cold acetone for 15 seconds. Wells were air-dried for 10 minutes and incubated with 1 μg/ml DAPI in PBS [140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.2)]/BSA (2 mg/ml) for 1 minute. Slides were mounted in Vectashield (Vector Laboratories, USA), sealed with nail polish, and subsequently analyzed with a Nikon Optiphot at 1,000 X magnification. For quantification, at least 100 cells were examined in each of three independent experiments for both control and stressed cells. Means and standard deviation (SD) are shown for the different experiments. Imaging with a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss MicroImaging Inc., Germany) was carried out with a 100 X objective and a series of z-sections of 0.5 μm was collected.

Indirect immunofluorescence microscopy—Control or stressed cells were fixed with 3.7% formaldehyde for 20 minutes at room temperature, collected by centrifugation, washed twice with P-solution and digested with 0.3-2 μg/μl zymolase at 30°C for 1 hour. Following two washes in P-solution, cells were immobilized on polylysine-coated slides, incubated with 0.1% Triton X-100 in PBS/BSA (2 mg/ml) for 5 minutes at room temperature and treated with ice-cold methanol for 6 minutes followed by ice-cold acetone for 30 seconds. Air-dried samples were blocked with PBS/BSA for 1 hour and incubated overnight with primary antibodies against the HA-epitope (diluted 1:500 in PBS/BSA, monoclonal antibody 12CA5, BaBCo, USA) at room temperature. Wells were washed three times with PBS/BSA and bound primary antibodies were detected with Cy3- or FITC-conjugated secondary antibodies (Jackson ImmunoResearch, USA). DNA was visualized by incubation with 1 μg/ml DAPI in PBS/BSA for 1 minute. Slides were mounted in Vectashield and sealed with nail polish. Samples were analyzed as described in the previous section.

Western blot analysis—Control or ethanol treated cells were collected by centrifugation and lysed by vortexing in 2 X gel sample buffer [2 mM DTT, 20%

(v/v) glycerol, 100 mM Tris-HCl (pH 6.8), 0.2% (w/v) Bromophenol Blue, 4% (w/v) SDS] and glass beads for 10 minutes at 95°C. Crude lysates were cleared by 5 minutes centrifugation (13,000 rpm, microfuge, room temperature) and separated by SDS-polyacrylamide gel electrophoresis. Proteins were blotted onto nitrocellulose membranes (Bio-Rad, USA). Filters were blocked one hour at room temperature in Tris-buffered saline (TBS) [137 mM NaCl, 20 mM Tris-HCl (pH 8.0)] containing 0.2% (v/v) Tween-20 or PBS containing 0.1% (v/v) Tween-20 and 5% non-fat dried milk and incubated overnight at 4°C with primary antibodies. Mouse polyclonal antibodies against Nmd5p were generated and affinity-purified essentially as described [92]. Primary antibodies against the following proteins or epitope tags were used: GFP (diluted 1:200, Clontech, USA), His6-epitope (diluted 1:100, Santa Cruz Biotechnology, USA), phosphoserine (diluted 1:200, Stressgen Bioreagents Corp., Canada), phosphothreonine (diluted 1:100, Stressgen Bioreagents Corp.), phosphotyrosine (diluted 1:2000, Stressgen Bioreagents Corp.), HA-tag (diluted 1:1000, clone 12CA5), affinity purified polyclonal antibodies against Nmd5p or mAb414 (diluted 1:1000, BaBCo, USA). Bound primary antibodies were detected with a 1:4000 dilution of goat anti-mouse or anti-rabbit IgG coupled to HRP (Jackson ImmunoResearch) for 2 hours at room temperature. Immunoreactive proteins were visualized with an ECL kit (Amersham Biotech, Canada).

Immobilized metal affinity chromatography (IMAC) and quantification of ECL signals— Ethanol-treated and control cells were collected by centrifugation, washed in large volumes of cold water and sediments were stored at -70°C. Cells were lysed with 10 mM Hepes (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM NaN_3, 1 mM β-mercaptoethanol by vortexing with glass beads at room temperature. Crude lysates obtained after centrifugation (13,000 rpm, 5 minutes, microfuge) were diluted 1:3 in binding buffer [100 mM sodium acetate (pH 3.3), 0.02% NaN_3]. Aliquot of the samples were used for SDS-PAGE ("Starting material"). Supernatants were loaded onto 1.5 ml IMAC resin (TALON, Clontech, USA) activated with 108 mg $FeCl_3$ in 4 ml distilled water and equilibrated in binding buffer. The resin was washed with 6 ml binding buffer and bound material was eluted in 20 mM sodium phosphate (pH 7.0)/0.02% NaN_3. Upon addition of 0.2 mg/ml deoxycholate to each fraction, samples were incubated for 30 minutes on ice. Proteins were precipitated with 6% TCA for 1 hour on ice, collected by centrifugation and resuspended in 2 X IMAC sample buffer [40 mM Tris-HCl (pH 8.8), 40% (v/v) glycerol, 0.2% (w/v) Bromophenol Blue, 4% (w/v) SDS, 1 mM EDTA, 2% (v/v) β-mercaptoethanol, 100 mM NaF, 40 mM Na_3VO_3, 600 mM β-glycerophosphate, 1 X protease inhibitor cocktail (Roche, Canada)]. Samples were heated for 10 minutes at 95°C, separated by SDS-PAGE and analyzed by western blotting as described above. Signals obtained after ECL were quantified by densitometry of the films using the software "Spot Density Tools" (Alpha Innotech Corporation, San Leandro, USA). Density measurements were carried out for each area of interest and corrected for background. All of the signals quantified for an individual experiment were obtained from the same filter.

Purification of Nmd5p-His6 from yeast cells under denaturing conditions—Yeast cell sediments were prepared and stored at -70°C as described for IMAC. Cells were lysed with glass beads/lysis buffer [0.1 M NaH_2PO_4/10 mM Tris (pH 8.0), 1 mM NaN_3, 0.5% Triton X-100, 1 mM β-mercaptoethanol, 1 X protease inhibitor cocktail, 8 M urea] by vortexing at room temperature. Crude lysates obtained after centrifugation (13,000 rpm, 5 minutes, microfuge) were incubated with Ni-NTA agarose (Qiagen, Germany) with gentle agitation for 1 hour at room temperature. The resin was collected by centrifugation, washed twice with lysis buffer and three times with lysis buffer without Triton X-100 or urea. Bound material was eluted by boiling for 10 min at 95°C with 2 X gel sample buffer containing 0.1 M EDTA, 2% (v/v) β-mercaptoethanol, 100 mM NaF, 40 mM Na_3VO_3, 600 mM β-glycerophosphate and 1 X protease inhibitor cocktail, followed by western blot analysis.

Immunoprecipitation of threonine-phosporylated proteins from yeast cells— Yeast cells were cultured, collected and stored as sediments at -70°C (as described above for IMAC). Cells were lysed with glass beads/Buffer A [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM NaN_3, 0.2% SDS, 1 mM Na_3VO_4, 1 mM DTT, 1 mM PMSF, 1 X protease inhibitor cocktail] by vortexing 15 minutes at 95°C. Crude cell lysates obtained after centrifugation were diluted 1:2 in Buffer B [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM NaN_3, 1 mM Na_3VO_4, 1% Nonidet P-40, 1 mM PMSF, 1 X protease inhibitor cocktail] and preincubated with protein G-Sepharose (Amersham Biotech, Canada) for 30 minutes at 4°C with gentle agitation. Supernatants obtained after 5 minutes centrifugation (10,000 rpm, microfuge, 4°C) were incubated with antibodies against phosphothreonine for at least 2 hours followed by addition of protein G-Sepharose and overnight incubation at 4°C. The resin was collected by centrifugation, washed twice with buffer B and once with buffer B without Nonidet P-40. Proteins bound to the resin were eluted by heating with 2 X gel sample buffer for 10 min at 95°C, followed by western blot analysis with antibodies against the HA-epitope, Nmd5p or phosphothreonine.

Purification of GST- kinase fusion proteins from yeast cells— Yeast cells encoding a GST-tagged kinase [91] were grown overnight at 30°C in Ura drop-out medium containing 3% raffinose. At an OD_{600} of 0.3-0.5, cells were collected by centrifugation (3000 g, 10 minutes at 4°C) and resuspended in Ura drop-out media containing 3% raffinose and 3% galactose. GST-kinases were synthesized during a 2-3 hours period of growth (30°C) until an OD_{600} of 0.8-1.0 was reached. Cells were collected by centrifugation (3000 g, 10 minutes at 4°C), washed in a large volume of cold water and stored at -70°C. Cells were resuspended in G-buffer [50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 50 mM NaF, 1 mM EDTA, 1% Triton X-100, 1 mM benzamidine, 0.1 mM PMSF, 2 X protease inhibitor cocktail] and lysed with pre-chilled glass beads by vortexing for 15-20 minutes at 4°C. The cell lysate was collected by centrifugation (3000 g, 10 minutes at 4°C) and incubated with glutathione-Sepharose beads with gentle agitation for 1 hour at 4°C. The resin was collected by centrifugation (500 g, 5 minutes at 4°C) and washed four times with G-buffer. GST fusion proteins were eluted from the beads with 50 mM Tris-HCl (pH 8.0)/10 mg/ml reduced glutathione by gentle agitation for 30 minutes at 4°C. Proteins were concentrated and buffer was exchanged to 20 mM Hepes (pH 7.4)/250 mM KCl/20% (v/v) glycerol with Amicon Ultra-4 filters (10,000 MW cut-off, Millipore, Canada). Samples were stored at -70°C until use.

2.4 Results

2.4.1 The cell integrity MAPK cascade is involved in targeting the N-terminal portion of Ssa4p to the nuclei of ethanol-treated cells.

2.4.1.1 Nuclear import of Ssa4p(1-236)-GFP upon ethanol exposure is reduced in different deletion mutants of the cell integrity MAPK cascade, but not in a deletion mutant lacking the downstream kinase Mck1p.

To determine the possible role of the cell integrity MAPK cascade in targeting the N-terminal portion of Ssa4p to the nuclei of ethanol-treated cells, we used the GFP-tagged reporter protein Ssa4p(1-236)-GFP [50, 85]. Its distribution in different deletion mutants of the cell integrity MAPK cascade was monitored by fluorescence microscopy. As shown in Fig. 3 and Fig. 4, Ssa4p(1-236)-GFP located throughout the cytoplasm and nuclei in wild type cells under normal growth conditions, but concentrated in the nuclei following 2 minutes of treatment with 10% ethanol. The number of wild type cells displaying Ssa4p(1-236)-GFP nuclear accumulation (N>C) upon ethanol treatment was increased to about 10 times the number obtained for unstressed cells (Fig. 4B). By contrast, deletion of any of the components of the cell integrity MAPK cascade reduced the nuclear accumulation of Ssa4p(1-236)-GFP upon ethanol exposure (Fig. 4A, N>C, yellow bars and Fig. 4B). In mutant strains, the number of cells with Ssa4p(1-236)-GFP nuclear accumulation after ethanol treatment increased to a lesser extent when compared to unstressed cells (Fig. 4B).

To further study the role of the cell integrity signaling pathway, I localized Ssa4p(1-236)-GFP in mutant strain *mck1*Δ. Mck1p is a direct downstream factor of the cell integrity MAPK cascade and a protein serine/threonine/tyrosine kinase which plays an important role in meiosis and mitosis [65, 93-95]. As shown in Fig. 4B, the relative increase of Ssa4p(1-236)-GFP nuclear accumulation upon ethanol treatment in *mck1*Δ was similar to that in wild type cells, which indicates that a functional Mck1p is not essential for Ssa4p(1-236)-GFP nuclear accumulation in ethanol-stressed cells.

Taken together, these data are consistent with the idea that the cell integrity MAPK cascade, but not the downstream kinase Mck1p, are important to the control of Ssa4p nuclear transport in ethanol-treated cells.

2.4.1.2 Ethanol treatment increases the cytoplasmic localization of Nmd5p-His6-HA in *pkc1*Δ, *bck1*Δ and *slt2*Δ mutants.

The cell integrity Pkc1p-MAPK cascade could affect Ssa4p(1-236)-GFP nuclear import in ethanol-stressed cells by different mechanisms. This could involve the cargo, carrier or components of the NPC. One possibility is that the cell integrity Pkc1p-MAPK cascade regulates Ssa4p(1-236)-GFP nuclear import in ethanol-treated cells by changing the localization of the carrier Nmd5p. Such a regulation could affect the availability of the carrier protein to its cargos. To address this point, I localized Nmd5p-His6-HA in different deletion mutants of the cell integrity pathway by immunofluorescence microscopy with antibodies against the HA-tag. As shown in Fig. 5, Nmd5p-His6-HA distributed throughout the cytoplasm and nuclei in wild type cells both under normal growth conditions and after treatment with 10% ethanol. However, in cells lacking functional Pkc1p, Bck1p or Slt2p, the amount of Nmd5p-His6-HA in the cytoplasm was drastically increased (Fig. 6). This change could result from the inhibition of Nmd5p nuclear import or the increase of its nuclear export in mutant strains. In *mkk1*Δ or *mkk2*Δ mutants, most of Nmd5p-His6-HA localized to the cytoplasm under normal growth conditions, and ethanol treatment did not alter drastically this distribution (Fig. 5 and Fig. 6).

On the basis of the above data, we conclude that the cell integrity Pkc1p-MAPK cascade is involved in targeting the N-terminal portion of Ssa4p to the nuclei of ethanol-treated cells. The localization and availability of Nmd5p may play a role in this process.

2.4.2 Phosphorylation of the carrier Nmd5p increases in ethanol-treated cells.

Phosphorylation may alter the electrophoretic mobility of a protein in

SDS-polyacrylamide gels, resulting in reduced migration of the modified protein during electrophoresis [95a, 95b]. To test the hypothesis that phosphorylation of the Ssa4p(1-236)-GFP transport machinery or NPC contributes to the nuclear accumulation of the hsp70 in ethanol-treated cells, the possible modification of the cargo protein Ssa4p(1-236)-GFP, the carrier Nmd5p and the nuleoporin Nsp1p were analyzed.

To determine whether Ssa4p(1-236)-GFP or components of the transport machinery change their migration during SDS-PAGE, I examined Ssa4p(1-236)-GFP, Nmd5p and the essential nucleoporin Nsp1p in control and ethanol-treated cells. Proteins were identified by western blotting with antibodies against GFP for Ssa4p(1-236)-GFP, the His6-tag for Nmd5p-His6 or mAb414, an antibody recognizing FXF repeats, for Nsp1p. As Pkc1p and the cell integrity MAPK cascade are crucial to Ssa4p(1-236)-GFP nuclear accumulation, we focused on mutants missing one of the components in this pathway. Such mutant strains were compared with wild type cells, and an additional control was included with wild type cells that synthesize the GFP-tag only.

As shown in Fig. 7A, no obvious band shift was seen for Ssa4p(1-236)-GFP, Nmd5p-His6 or Nsp1p in any of the knock-out strains of the cell integrity Pkc1p-MAPK cascade and control cells. However, these experiment do not rule out an ethanol-induced modification of any of the proteins since the molecular mass of Ssa4p(1-236)-GFP (~50 kDa), Nmd5p-His6 (~120 kDa) and Nsp1p (~90 kDa) are far larger than that of phosphate (~80 Da). Thus, the addition of one or few phosphates may not be detected with this assay.

Therefore, I further monitored the possible phosphorylation of Ssa4p(1-236)-GFP, Nmd5p-His6 and Nsp1p using immobilized metal affinity chromatography (IMAC) as an independent approach. To achieve this, phosphoproteins were isolated from control or ethanol-treated cells and analyzed by western blotting with specific antibodies. Fig. 7B (right panel) demonstrates that the carrier Nmd5p, its cargo Ssa4p(1-236)-GFP and the nucleoporin Nsp1p can be purified by IMAC both from control and ethanol-treated cells. However, only Nmd5p phosphorylation changed drastically in ethanol-treated cells (P=0.07) (Fig.7B left panel); the amount of Nmd5p-His6 purified by IMAC increased approximately 2-fold upon exposure to ethanol.

2.4.3 Nmd5p is phosphorylated on threonine residues.

Phosphospecific antibodies were used to determine the phosphorylation status of Nmd5p and identify amino acid side chains that are modified in Nmd5p. Nmd5p-His6-HA was purified from crude cell extracts under denaturing conditions and proteins were separated by SDS-PAGE. Blots were probed with antibodies recognizing phosphoserine, phosphothreonine or phosphotyrosine, respectively (Fig. 7C). A weak signal was detected for phosphothreonine for a band of ~120 kDa (Fig. 7C-a) and smaller proteins, likely degradation products of the carrier Nmd5p. By contrast, serine and tyrosine phosphorylation was not detected for Nmd5p with this assay (Fig. 7C-c). To further confirm the phosphorylation of threonine residue(s) in Nmd5p, I first isolated proteins by indirect immunoprecipitation with phosphothreonine specific antibodies followed by western blot analyses. Equal amounts of immunoprecipitates were loaded on two halves of the same filter. One half of the filter was probed with antibodies against phosphothreonine to monitor the efficiency of immunoprecipitation (data not shown). The other portion was incubated with primary antibodies against the HA-tag to detect Nmd5p-His6-HA. Furthermore, the filter was stripped and reprobed with affinity-purified antibodies against Nmd5p. These experiments confirmed the phosphorylation of threonine residue(s) *in vivo* for the carrier Nmd5p-His6-HA. It should be noted that in our studies, Nmd5p was proteolytically degraded and a predominant product of ~60 kDa was obtained after immunoprecipitation (Fig.7C-b).

2.4.4 In a *snf1*Δ mutant Ssa4p(1-236)-GFP fails to accumulate in nuclei upon ethanol stress. This phenotype can be partially rescued by overexpression of GST-Snf1p.

We were interested in the role of Snf1p kinase in Ssa4p(1-236)-GFP nuclear import for several reasons. First, unpublished data (not shown here) from our group demonstrate that Ssa4p(1-236)-GFP fails to accumulate in nuclei of a *snf1*Δ mutant upon ethanol stress. Second, Nmd5p contains the sequence LLRDI*T*PISW (residues 704-713) that is similar to the consensus for Snf1p kinase [69, 72-75]. Interestingly, the potential Snf1p site corresponding to threonine709 of Nmd5p in *S. cerevisiae* is conserved as threonine or serine among different species (Fig. 8) [96]. Third, the phosphorylation status of the carrier Nmd5p changes with ethanol stress, and phosphothreonine was detected in Nmd5p (see above). On the basis of these results, I localized Ssa4p(1-236)-GFP in cells lacking a functional Snf1p kinase. My studies confirmed that Ssa4p(1-236)-GFP localizes throughout the cytoplasm and nucleus in *snf1*Δ mutant cells, even after exposure to ethanol stress (Fig 9A). I next transformed plasmids encoding Ssa4p(1-236)-GFP as well as GST-Snf1p, respectively, into *snf1*Δ cells. In these experiments, cell viability was drastically reduced by the overexpression of *GST-SNF1*. Nevertheless, it was obvious that *GST-SNF1* overexpression can partially rescue ethanol-induced nuclear import of Ssa4p(1-236)-GFP in *snf1*Δ cells (Fig. 9B). Taken together, my experiments demonstrate that Snf1p is involved in concentrating Ssa4p(1-236)-GFP in nuclei of ethanol-stressed cells.

2.5 Discussion and conclusions

In eukaryotic cells, the nuclear envelope separates nucleus and cytoplasm. Communication between the two subcellular compartments is essential for maintaining a large number of cellular activities. Regulation of protein transport in and out of the nucleus plays a pivotal role in these processes. Our group reported previously that Ssa4p, a cytoplasmic hsp70 in budding yeast, translocates to nuclei when cells are exposed to ethanol. Under the same conditions, classical nuclear import of most proteins is inhibited [85]. The N-terminal 236 amino acid residues of Ssa4p are sufficient to target the passenger protein GFP to nuclei in ethanol-exposed cells, and Nmd5p is the karyopherin-β protein that carries Ssa4p into nuclei [85]. Furthermore, Ssa4p(1-236)-GFP fails to accumulate in nuclei when mutants lacking Wsc1p, Mid2p or protein kinase C (Pkc1p) are exposed to ethanol [85].

Wsc1p and Mid2p are two of the major cell surface sensors of the yeast cell integrity pathway; both are located in the plasma membrane. Pkc1p is involved in several signaling pathways including the MAPK cascade of the cell integrity pathway, which is composed of a linear series of protein kinases including a MAP kinase kinase kinase (Bck1p), a pair of redundant MAP kinase kinases (Mkk1p and Mkk2p), and a MAP kinase (Slt2p) [62, 63]. To identify a possible link between the regulation of Ssa4p(1-236)-GFP nuclear import and the cell integrity

Pkc1p-MAPK cascade, I have localized Ssa4p(1-236)-GFP in ethanol-treated yeast cells that are lacking one of the functional components of the cell integrity MAPK cascade. With these experiments, we have shown that ethanol drastically reduces the nuclear accumulation of Ssa4p(1-236)-GFP in different mutants of the cell integrity MAPK cascade. Bck1p and Slt2p are the only MAPK kinase kinase and MAPK of the cell integrity signaling pathway and knocking out either kinase will abolish the function of the cascade. As for Mkk1p and Mkk2p, the pair of redundant MAPK kinases in the cascade, in previous studies functional inactivation of either kinase did not cause an apparent abnormal phenotype, and only the simultaneous inactivation of both genes resulted in a *slt2*Δ-like and *bck1*Δ-like phenotype [61]. However, for the data presented in this thesis a knock-out of either *MKK1* or *MKK2* reduced ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP. This reduction in nuclear accumulation was similar to what was observed in *slt2*Δ cells, suggesting that the functions of Mkk1p and Mkk2p are not completely overlapping. One possible explanation is that both kinase activities may be required under specific physiological conditions including the response to ethanol.

Taken together, my studies have demonstrated that the cell integrity MAPK pathway is involved in ethanol-induced Ssa4p(1-236)-GFP nuclear accumulation. Nmd5p is the nuclear importer for Ssa4p(1-236)-GFP in ethanol-treated cells [85] and this carrier may play a role in the regulation of Ssa4p(1-236)-GFP nuclear import. For instance, the availability of Nmd5p to Ssa4p(1-236)-GFP could control import of its cargo. If this is the case, one might speculate that the Pkc1p-cell integrity pathway is a link between Ssa4p(1-236)-GFP nuclear accumulation and the availability of the carrier. To address this point, we have monitored the distribution of Nmd5p-His6-HA in different deletion mutants of the cell integrity Pkc1p-MAPK cascade upon ethanol stress. Consistent with the results from other groups Nmd5p-His6-HA localizes throughout cytoplasm and nucleus in wild type cells under normal growth conditions [97, 98]. Previous reports have shown that ethanol or sodium chloride exposure concentrates Nmd5p-GFP at the nuclear envelope in some yeast strains [85, 98]. However, this was not observed in the experiments described here, and there are several possible explanations that will have to be investigated in future studies. First, the nuclear accumulation of Nmd5p-GFP in response to ethanol or osmotic stress could depend on the strain background. Second, for the studies described here synthesis of Nmd5p-His6-HA was controlled by a *GALS* promoter, and overexpression of the gene may have masked a concentration of Nmd5p-His6-HA at the nuclear envelope. Nevertheless, my experiments revealed that the Pkc1p-cell integrity pathway is implicated in the nucleocytoplasmic distribution of the carrier Nmd5p. Unlike wild type cells, *pkc1*Δ, *bck1*Δ and *slt2*Δ strains showed increased levels of

Nmd5p-His6-HA in the cytoplasm after incubation with ethanol. Upon ethanol treatment we did not detect the carrier in nuclei of the mutant strains; but without ethanol, Nmd5p-His6-HA was clearly associated with the nucleus. These data suggest that Pkc1p, Bck1p and Slt2p may have a role in regulating the distribution of Nmd5p which might restrict the amount of Nmd5p available to its cargo Ssa4p(1-236)-GFP. Ultimately, this could result in the mislocalization of Ssa4p(1-236)-GFP in ethanol-stressed mutant strains. Alternatively, a change in the distribution of Nmd5p may represent a defect of the carrier to translocate across the NPC in ethanol-treated *pkc1*Δ, *bck1*Δ and *slt2*Δ cells, subsequently leading to the mislocalization of Ssa4p(1-236)-GFP. The mechanism underlying the increase in cytoplasmic Nmd5p-His6-HA in ethanol-treated mutant strains is at present not understood. It could be the outcome of increased nuclear export of Nmd5p-His6-HA or decreased nuclear import of Nmd5p-His6-HA, or a combination of both.

Unlike *bck1*Δ and *slt2*Δ, we did not observe that the amount of Nmd5p-His6-HA was reduced in nuclei of ethanol-stressed *mkk1*Δ and *mkk2*Δ mutants. As discussed above, Mkk1p and Mkk2p have overlapping functions and either kinase may be sufficient to ensure the proper localization of Nmd5p-His6-HA. However, lacking a functional Mkk1p or Mkk2p kinase does reduce the ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP as shown in this thesis. The combination of our results obtained for Ssa4p(1-236)-GFP and Nmd5p-His6-HA may indicate that Mkk1p and Mkk2p do play a role in the correct localization of Ssa4p(1-236)-GFP. However, we propose that Mkk1p and Mkk2p affect a yet to be defined reaction that is different from the localization of Nmd5p in nuclei of ethanol-stressed cells.

In conclusion, the cell integrity Pkc1p-MAPK pathway is involved in the ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP. Failure to concentrate Ssa4p(1-236)-GFP in nuclei of ethanol-treated cells correlates with the decreased level of Nmd5p in nuclei. This mislocalization of Nmd5p may partially contribute to the reduced levels of Ssa4p(1-236)-GFP detected in the nucleus of mutant cells after incubation with ethanol.

Having shown that the cell integrity Pkc1p-MAPK pathway participates in nuclear accumulation of Ssa4p(1-236)-GFP in ethanol-treated cells, we have begun to analyze the possible role of downstream factors of the MAPK cascade in this process. So far, only few downstream targets have been identified for Slt2p. This includes Mck1p, a yeast homologue of mammalian glycogen synthase kinase 3. To study the contribution of Mck1p in Ssa4p nuclear import, Ssa4p(1-236)-GFP was localized in ethanol-treated *mck1*Δ cells. In these experiments, ethanol-stressed *mck1*Δ cells concentrate Ssa4p(1-236)-GFP in nuclei similar to the wild type (Fig. 4B). Therefore, Mck1p is unlikely to play an essential role in the ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP.

As discussed in the Chapter I, the dynamic phosphorylation of components of the transport apparatus is crucial to the regulation of nuclear protein trafficking. Hence, the potential post-translational modification of Ssa4p(1-236)-GFP, its carrier Nmd5p or Nsp1p, an essential nucleoporin involved in many nuclear transport pathways, was analyzed. Furthermore, it was determined whether phosphorylation is altered in response to ethanol treatment. Our data indicate that the phosphorylation status of Nmd5p changes when cells are treated with ethanol, and threonine residue(s) are putative targets for this modification.

In addition to Ssa4p [85], Nmd5p has been identified as the import receptor of HOG1 MAP kinase [98], transcription factors Crz1p [99] and TFIIS [97]. Nmd5p shows a high affinity for HOG1 MAP kinase only when HOG1 is in a phosphorylated form. However, only dephosphorylated Crz1p can be transported into nuclei by Nmd5p. In our studies, the control of Nmd5p binding to Ssa4p(1-236)-GFP could be regulated through the phosphorylation of the carrier itself, rather than the cargo Ssa4p, although we have not excluded the possibility that modification of Ssa4p contributes to its localization.

To further study the ethanol-induced modification of Nmd5p, we have begun to define the kinase(s) responsible for phosphorylation of Nmd5p in ethanol-stressed cells. Interestingly, protein sequence analysis shows that Nmd5p contains a consensus site for phosphorylation by Snf1p kinase. In addition, our results demonstrate that ethanol fails to induce nuclear accumulation of Ssa4p(1-236)-GFP in cells lacking a functional Snf1p kinase; this phenotype can be partially rescued by overexpression of *GST-SNF1* in *snf1*Δ cells. Future experiments will have to determine whether Snf1p phosphorylates Nmd5p upon ethanol stress, and whether this contributes to the regulation of Nmd5p function. In summary, my studies suggest that phosphorylation of the carrier Nmd5p could play a role in nuclear accumulation of Ssa4p(1-236)-GFP in ethanol-treated cells. My results point to threonine residue(s) as a possible target for phosphorylation. Moreover, Snf1p is a candidate kinase which may be responsible for the phosphorylation of Nmd5p in response to ethanol stress.

The results described in my thesis set the stage for future studies that will focus on the following aspects: First, *in vitro* kinase assays will be carried out to determine the candidate kinase(s) that modify Nmd5p in response to ethanol exposure. By incubating Nmd5p purified from *E. coli* with the candidate kinase and [γ-^{32}P]-ATP, kinase(s) which directly phosphorylate Nmd5p *in vitro* could be identified. Since the cell integrity Pkc1p-MAPK cascade and Snf1p kinase are involved in ethanol-induced Ssa4p(1-236)-GFP nuclear accumulation, these *in vitro* assays will initially focus on Pkc1p, kinases of the cell integrity pathway and Snf1p. Most of the yeast kinases are available to us as GST-fusions [91] and testing all of these enzymes *in vitro* may identify additional candidate kinases that modify not only Nmd5p, but also Ssa4p or Nsp1p. Once we have preliminary data for the candidate kinase(s) from *in vitro* assays, *in vivo* studies will be carried out to determine whether any of the candidate kinase(s) are relevant for Ssa4p nuclear transport in growing cells.

Second, to define the target residue(s) modified in Nmd5p in response to ethanol treatment, site-directed mutagenesis of the candidate phosphorylation site(s) will be carried out. Furthermore, the localization of a mutant Nmd5p that carries a constitutive negative charge at the putative site to mimic phosphorylation, or a non-phosphorylatable residue could define how phosphorylation at a particular site(s) affects nuclear trafficking of the carrier.

Third, to determine whether Nmd5p phosphorylation is required for nuclear accumulation of Ssa4p in ethanol-stressed cells, one could introduce a mutant allele of *NMD5* into *nmd5Δ* cells and monitor the distribution of Ssa4p(1-236)-GFP with or without ethanol treatment.

In conclusion, research presented here and future studies based on my results will allow us to better understand the mechanism of hsp70s nuclear trafficking. Moreover, it will help to define the regulation of Ssa4p nucleocytoplasmic transport in response to various signals that control cell physiology under normal and stress conditions.

References

[1] Fahrenkrog, B., and Aebi, U. (2003) The nuclear pore complex: nucleocytoplasmic transport and beyond. *Nat. Rev. Mol. Cell Biol.* 4, 757-766.

[2] Davis, L. I. (1995) The nuclear pore complex. *Annu. Rev. Biochem.* 64, 865-896.

[3] Beck, M., Forster, F., Ecke, M., Plitzko, J. M., Melchior, F., Gerisch, G., Baumeister, W., and Medalia, O. (2004) Nuclear pore complex structure and dynamics revealed by cryoelectron tomography. *Science* 306, 1387-1390.

[4] Suntharalingam, M., and Wente, S. R. (2003) Peering through the pore: Nuclear pore complex structure, assembly, and function. *Dev. Cell.* 4, 775-789.

[5] Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y., and Chait, B. T. (2000) The yeast nuclear pore complex: composition, architecture, and transport mechanism. *J. Cell Biol.* 148, 635-652.

[6] Rout, M. P., and Aitchison, J. D. (2001) The nuclear pore complex as a transport machine. *J. Biol. Chem.* 276, 16593-16596.

[7] Pante, N. (2004) Nuclear pore complex structure: unplugged and dynamic pores. *Dev. Cell.* 7, 780-781.

[8] Ohno, M., Fornerod, M., and Mattaj, I. W. (1998) Nucleocytoplasmic transport: The last 200 nanometers. *Cell* 92, 327-336.

[9] Kubitscheck, U., Grunwald, D., Hoekstra, A., Rohleder, D., Kues, T., Siebrasse, J. P., and Peters, R. (2005) Nuclear transport of single molecules: Dwell times at the nuclear pore complex. *J. Cell Biol.* 168, 233-243.

[10] Antonin, W., and Mattaj, I. W. (2005) Nuclear pore complexes: Round the bend? *Nat. Cell Biol.* 7, 10-12.

[11] Adam, S. (2001) The nuclear pore complex. *Genome Biol.* 2, reviews0007.1 - reviews0007.6.

[12] Bednenko, J., Cingolani, G., and Gerace, L. (2003) Nucleocytoplasmic transport: Navigating the channel. *Traffic* 4, 127-135.

[13] Fried, H., and Kutay, U. (2003) Nucleocytoplasmic transport: Taking an inventory. *Cell Mol. Life Sci.* 60, 1659-1688.

[14] Stochaj, U., Rother, K. L. (1999) Nucleocytoplasmic trafficking of proteins: With or without Ran? *BioEssays* 21, 579-589.

[15] Pemberton, L. F., and Paschal, B. M. (2005) Mechanisms of receptor-mediated nuclear import and nuclear export. *Traffic* 6, 187-198.

[16] Goldfarb, D. S., Corbett, A. H., Mason, D. A., Harreman, M. T., and Adam, S. A. (2004) Importin α: a multipurpose nuclear-transport receptor. *Trends Cell Bio.* 14, 505-514.

[17] Robbins, J., Dilwortht, S. M., Laskey, R. A., and Dingwall, C. (1991) Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: Identification of a class of bipartite nuclear targeting sequence. *Cell* 64, 615-623.

[18] Görlich, D., and Kutay, U. (1999) Transport between the cell nucleus and the cytoplasm. *Annu. Rev. Cell Dev. Biol.* 15, 607-660.

[19] Kuersten, S., Ohno, M., and Mattaj, I. W. (2001) Nucleocytoplasmic transport: Ran, beta and beyond. *Trends Cell Biol.* 11, 497-503.

[20] Nakielny, S., and Dreyfuss, G. (1999) Transport of proteins and RNAs in and out of the Nucleus. *Cell* 99, 677-690.

[21] Macara, I. G. (2001) Transport into and out of the nucleus. *Microbiol. Mol. Biol. Rev.* 65, 570-594.

[22] Marelli, M., Dilworth, D. J., Wozniak, R. W., and Aitchison, J. D. (2001) The dynamics of karyopherin-mediated nuclear transport. *Biochem. Cell Biol.* 79, 603-612.

[23] Mosammaparast, N., and Pemberton, L. F. (2004) Karyopherins: from nuclear-transport mediators to nuclear-function regulators. *Trends Cell Biol.* 14, 547-556.

[24] Hood, J. K., and Silver, P. A. (1999) In or out? Regulating nuclear transport. *Curr. Opin. Cell Biol.* 11, 241-247.

[25] Kaffman, A., and O'Shea, E. K. (1999) Regulation of nuclear localization: A key to a door. *Annu. Rev. Cell Dev. Biol.* 15, 291-339.

[26] Nigg, E. A. (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature* 386, 779-787.

[27] Hubner, S., Xiao, C.-Y., and Jans, D. A. (1997) The protein kinase CK2 site (Ser111/112) enhances recognition of the Simian Virus 40 large T-antigen nuclear localization sequence by importin. *J. Biol. Chem.* 272, 17191-17195.

[28] Longshaw, V. M., Chapple, J. P., Balda, M. S., Cheetham, M. E., and Blatch, G. L. (2004) Nuclear translocation of the hsp70/hsp90 organizing protein mSTI1 is regulated by cell cycle kinases. *J. Cell Sci.* 117, 701-710.

[29] Kawajiri, K., and Ikuta, T. (2004) Regulation of nucleo-cytoplasmic transport of the aryl hydrocarbon receptor. *J. Health. Sci.* 50, 215-219.

[30] Liang, S.-H., and Clarke, M. F. (2001) Regulation of p53 localization. *Eur. J. Biochem.* 268, 2779-2783.

[31] Sheng, T., Chi, S., Zhang, X., and Xie, J. (2006) Regulation of Gli1 localization by the cAMP/protein kinase A signaling axis through a site near the nuclear localization signal. *J. Biol. Chem.* 281, 9-12.

[32] New, L., Jiang, Y., and Han, J. (2003) Regulation of PRAK subcellular location by p38 MAP kinases. *Mol. Biol. Cell* 14, 2603-2616.

[33] Azuma, Y., Tabb, M. M., Vu, L., and Nomura, M. (1995) Isolation of a yeast protein kinase that is activated by the protein encoded by SRP1 (Srp1p) and phosphorylates Srp1p complexed with nuclear localization signal peptides. *Proc. Natl. Acad. Sci. USA.* 92, 5159-5163.

[34] Wang, W., Yang, X., Kawai, T., de Silanes, I. L., Mazan-Mamczarz, K., Chen, P., Chook, Y. M., Quensel, C., Kohler, M., and Gorospe, M. (2004) AMP-activated protein kinase-regulated phosphorylation and acetylation of importin α1: Involvement in the nuclear import of RNA-binding protein HuR. *J. Biol. Chem.* 279, 48376-48388.

[35] Li, M., Brooks, C. L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003) Mono- versus polyubiquitination: Differential control of p53 fate by Mdm2. *Science* 302, 1972-1975.

[36] Smith, W. A., Schurter, B. T., Wong-Staal, F., and David, M. (2004) Arginine methylation of RNA Helicase A determines its subcellular localization. *J. Biol. Chem.* 279, 22795-22798.

[37] Lee, P. S. W., Chang, C., Liu, D., and Derynck, R. (2003) Sumoylation of Smad4, the common Smad mediator of transforming growth factor-β family signaling. *J. Biol. Chem.* 278, 27853-27863.

[38] Görlich, D., Seewald, M. J., and Ribbeck, K. (2003) Characterization of Ran-driven cargo transport and the RanGTPase system by kinetic measurements and computer simulation. *EMBO J.* 22, 1088–1100.

[39] Lu, D., Yang, H., and Raizada, M. K. (1998) Involvement of p62 nucleoporin in angiotensin II-induced nuclear translocation of STAT3 in brain neurons. *J. Neurosci.* 18, 1329-1336.

[40] Kiang, J. G., and Tsokos, G. C. (1998) Heat shock protein 70 kDa: Molecular biology, biochemistry, and physiology. *Pharmacol. Ther.* 80, 183-201.

[41] Beere, H. M., and Green, D. R. (2001) Stress management - heat shock protein-70 and the regulation of apoptosis. *Trends Cell Bio.* 11, 6-10.

[42] Mager, W., and Ferreira, P. (1993) Stress response of yeast. *Biochem. J.* 290, 1-13.

[43] Morano, K. A., Liu, P. C. C., and Thiele, D. J. (1998) Protein chaperones and the heat shock response in *Saccharomyces cerevisiae. Curr Opin Microbiol.* 1, 197-203.

[44] James, P., Pfund, C., and Craig, E. A. (1997) Functional specificity among hsp70 molecular chaperones. *Science* 275, 387-389.

[45] Craven, R. A., Egerton, M., and Stirling, C. J. (1996) A novel hsp70 of the yeast ER lumen is required for the efficient translocation of a number of protein precursors. *EMBO J.* 15, 2640-2650.

[46] Shaner, L., Trott, A., Goeckeler, J. L., Brodsky, J. L., and Morano, K. A. (2004) The function of the yeast molecular chaperone Sse1 is mechanistically distinct from the closely related hsp70 family. *J. Biol. Chem.* 279, 21992-22001.

[47] Shaner, L., Wegele, H., Buchner, J., and Morano, K. A. (2005) The yeast hsp110 Sse1 functionally interacts with the hsp70 chaperones Ssa and Ssb. *J. Biol. Chem.* 280, 41262-41269.

[48] Horton, L. E., James, P., Craig, E. A., and Hensold, J. O. (2001) The yeast hsp70 homologue Ssa is required for translation and interacts with Sis1 and Pab1 on translating ribosomes. *J. Biol. Chem.* 276, 14426-14433.

[49] Schmidt, S., Strub, A., Rottgers, K., Zufall, N., and Voos, W. (2001) The two mitochondrial heat shock proteins 70, Ssc1 and Ssq1, compete for the cochaperone Mge1. *J. Mol. Biol.* 313, 13-26.

[50] Chughtai, Z. S., Rassadi, R., Matusiewicz, N., and Stochaj, U. (2001) Starvation promotes nuclear accumulation of the hsp70 Ssa4p in yeast cells. *J. Biol. Chem.* 276, 20261-20266.

[51] Craig, E. A., Gambill, B. D., and Nelson, R. J. (1993) Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol. Rev.* 52, 402–414.

[52] Hartl, F. U., and Hayer-Hartl, M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295, 1852-1858.

[53] Piper, P. W. (1995) The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiol. Lett.* 134, 121-127.

[54] Beere, H. M. (2005) Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J. Clin. Invest.* 115, 2633-2639.

[55] Morishima, N. (2005) Control of cell fate by hsp70: More than an evanescent meeting. *J. Biochem. (Tokyo)* 137, 449-453.

[56] Parsell, D. A., and Lindquist, S. (1993) The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* 27, 437-496

[57] Aufricht, C. (2005) Heat-shock protein 70: Molecular supertool? *Pediatr. Nephrol.* 20, 707-713.

[58] Mosser, D. D., Caron, A. W., Bourget, L., Meriin, A. B., Sherman, M. Y., Morimoto, R. I., and Massie, B. (2000) The chaperone function of hsp70 is required for protection against stress-induced apoptosis. *Mol. Cell. Biol.* 20, 7146-7159.

[59] Nollen, E. A. A., and Morimoto, R. I. (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing `heat shock' proteins. *J. Cell Sci.* 115, 2809-2816.

[60] Vujanac, M., Fenaroli, A., and Zimarino, V. (2005) Constitutive nuclear import and stress-regulated nucleocytoplasmic shuttling of mammalian heat-shock factor 1. *Traffic* 6, 214-229.

[61] Hohmann, S. (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* 66, 300-372.

[62] Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev.* 62, 1264-1300.

[63] Levin, D. E. (2005) Cell wall integrity signaling in *Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev.* 69, 262-291.

[64] Heinisch, J. J., Lorberg, A., Schmitz, H.-P., and Jacoby, J. J. (1999) The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in *Saccharomyces cerevisiae. Mol. Microbiol.* 32, 671-680.

[65] Mizunuma, M., Hirata, D., Miyaoka, R., and Miyakawa, T. (2001) GSK-3 kinase Mck1 and calcineurin coordinately mediate Hsl1 down-regulation by Ca2+ in budding yeast. *EMBO J.* 20, 1074-1085.

[66] Martin, H., Flandez, M., Nombela, C., and Molina, M. (2005) Protein phosphatases in MAPK signalling: we keep learning from yeast. *Mol. Microbiol.* 58, 6-16.

[67] Hardie, D. G., Carling, D., and Carlson, M. (1998) The AMP-activated/Snf1 protein kinase subfamily: Metabolic sensors of the eukaryotic cell? *Annu. Rev. Biochem.* 67, 821-855

[68] Rudolph, M. J., Amodeo, G. A., Bai, Y., and Tong, L. (2005) Crystal structure of the protein kinase domain of yeast AMP-activated protein kinase Snf1. *Biochem. Biophys. Res. Commun.* 337, 1224-1228.

[69] Sanz, P., Alms, G. R., Haystead, T. A. J., and Carlson, M. (2000) Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. *Mol. Cell Biol.* 20, 1321-1328.

[70] Liu, Y., Xu, X., Singh-Rodriguez, S., Zhao, Y., and Kuo, M.-H. (2005) Histone H3 Ser10 phosphorylation-independent function of Snf1 and Reg1 proteins rescues a gcn5- mutant in *HIS3* expression. *Mol. Cell Biol.* 25, 10566-10579.

[71] Vincent, O., Townley, R., Kuchin, S., and Carlson, M. (2001) Subcellular localization of the Snf1 kinase is regulated by specific β subunits and a novel glucose signaling mechanism. *Genes Dev.* 15, 1104-1114.

[72] Dale, S., Wilson, W. A., Edelman, A. M., and Hardie, D. G. (1995) Similar substrate recognition motifs for mammalian AMP-activated protein kinase, higher plant HMG-CoA reductase kinase-A, yeast SNF1, and mammalian calmodulin-dependent protein kinase I. *FEBS Lett.* 361, 191-195.

[73] Cziferszky, A., Seiboth, B., and Kubicek, C. P. (2003) The Snfl kinase of the filamentous fungus *Hypocrea jecorina* phosphorylates regulation-relevant serine residues in the yeast carbon catabolite repressor Mig1 but not in the filamentous fungal counterpart Cre1. *Fungal Genet. Biol.* 40, 166-175.

[74] Schüller, H.-J. (2003) Transcriptional control of nonfermentative metabolism in the yeast *Saccharomyces cerevisiae. Curr. Genet.* 43, 139-160.

[75] Charbon, G., Breunig, K. D., Wattiez, R., Vandenhaute, J., and Noel-Georis, I. (2004) Key role of Ser562/661 in Snf1-dependent regulation of Cat8p in *Saccharomyces cerevisiae* and *Kluyveromyces lactis. Mol. Cell. Biol.* 24, 4083-4091.

[76] Lo, W.-S., Duggan, L., Emre, N. C. T., Belotserkovskya, R., Lane, W. S., Shiekhattar, R., and Berger, S. L. (2001) Snf1-a histone kinase that works in concert with the histone acetyltransferase Gcn5 to regulate transcription. *Science* 293, 1142-1146.

[77] Rolland, F., Winderickx, J., and Thevelein, J. M. (2001) Glucose-sensing mechanisms in eukaryotic cells. *Trends Biochem. Sci.* 26, 310-317.

[78] Wilson, W. A., Hawley, S. A., and Hardie, D. G. (1996) Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr. Bio.* 6, 1426-1434.

[79] McCartney, R. R., and Schmidt, M. C. (2001) Regulation of Snf1 kinase. Activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. *J. Biol. Chem.* 276, 36460-36466.

[80] Sanz, P. (2001) Snf1 protein kinase: A key player in the response to cellular stress in yeast. *Biochem. Soc. Trans.* 31, 178–181.

[81] McCartney, R. R., Rubenstein, E. M., and Schmidt, M. C. (2005) Snf1 kinase complexes with different beta subunits display stress-dependent preferences for the three Snf1-activating kinases. *Curr. Genet.* 47, 335-344.

[82] Pelham, H. R. (1984) Hsp70 accelerates the recovery of nucleolar morphology after heat shock. *EMBO J.* 3, 3095–3100.

[83] Mandell, R. B., and Feldherr, C. M. (1990) Identification of two HSP70-related Xenopus oocyte proteins that are capable of recycling across the nuclear envelope. *J. Cell Biol.* 111, 1775-1783.

[84] Kodiha, M., Chu, A., Lazrak, O., and Stochaj, U. (2005) Stress inhibits nucleocytoplasmic shuttling of heat shock protein hsc70. *Am. J. Physiol. Cell Physiol.* 289, C1034-1041.

[85] Quan, X., Rassadi, R., Rabie, B., Matusiewicz, N., and Stochaj, U. (2004) Regulated nuclear accumulation of the yeast hsp70 Ssa4p in ethanol-stressed cells is mediated by the N-terminal domain, requires the nuclear carrier Nmd5p and protein kinase C. *FASEB J.* 899-901.

[86] Nanduri, J., and Tartakoff, A. M. (2001) The arrest of secretion response in yeast: Signaling from the secretory path to the nucleus via Wsc proteins and Pkc1p. *Mol. Cell.* 8, 281-289.

[87] Harreman, M. T., Kline, T. M., Milford, H. G., Harben, M. B., Hodel, A. E., and Corbett, A. H. (2004) Regulation of nuclear import by phosphorylation adjacent to nuclear localization signals. *J. Biol. Chem.* 279, 20613-20621.

[88] Jans, D. A., and Hubner, S. (1996) Regulation of protein transport to the nucleus: Central role of phosphorylation. *Physiol. Rev.* 76, 651-685.

[89] Lutfiyya, L. L., Iyer, V. R., DeRisi, J., DeVit, M. J., Brown, P. O., and Johnston, M. (1998) Characterization of three related glucose repressors and genes they regulate in *Saccharomyces cerevisiae. Genetics* 150, 1377-1391.

[90] Mitchell, D. A., Marshall, T. K., and Deschenes, R. J. (1993) Vectors for the inducible overexpression of glutathione S-transferase fusion proteins in yeast. *Yeast* 9, 715-722.

[91] Zhu, H., Klemic, J. F., Chang, S., Bertone, P., Casamayor, A., Klemic, K. G., Smith, D., Gerstein, M., Reed, M. A., and Snyder, M. (2000) Analysis of yeast protein kinases using protein chips. *Nat. Genet.* 26, 283-289.

[91a] Barth, W., and Stochaj, U. (1996) The yeast nucleoporin Nsp1 binds nuclear localization sequences *in vitro*. *Biochem. Cell Biol.* 74, 363–372.

[92] Stochaj, U., Osborne, M., Kurihara, T., and Silver, P. (1991) A yeast protein that binds nuclear localization signals: purification localization, and antibody inhibition of binding activity. *J. Cell Biol.* 113, 1243-1254.

[93] Hirata, Y., Andoh, T., Asahara, T., and Kikuchi, A. (2003) Yeast glycogen synthase kinase-3 activates Msn2p-dependent transcription of stress responsive genes. *Mol. Biol. Cell* 14, 302-312.

[94] Lim, M. Y., Dailey, D., Martin, G. S., and Thorner, J. (1993) Yeast MCK1 protein kinase autophosphorylates at tyrosine and serine but phosphorylates exogenous substrates at serine and threonine. *J. Biol. Chem.* 268, 21155-21164.

[95] Neigeborn, L., and Mitchell, A. P. (1991) The yeast Mck1 gene encodes a protein-kinase homolog that activates early meiotic gene-expression. *Genes Dev.* 5, 533-548.

[95a] Fleming, I.N., Elliott, C.M., Buchanan, F.G., Downes, C.P. and Exton, J.H. (1999) Ca^{2+}/calmodulin-dependent protein kinase II regulates Tiam1 by reversible protein phosphorylation. *J. Biol. Chem.* 274, 12753–12758.

[95b] Evans, J., Fergus, D., Leslie, C. (2002) Inhibition of the MEK1/ERK pathway reduces arachidonic acid release independently of cPLA2 phosphorylation and translocation. *BMC Biochem.* 3, 1471-2091

[96] The protein sequence of NMD5/YJR132W was used as the query for PSI-BLAST analysis against the UniRef90 protein dataset at UniProt.

<*http://db.yeastgenome.org/cgi-bin/homolog/uniprotHomolog?locus=YJR 132W*> (Last updated: November 2005).

[97] Albertini, M., Pemberton, L. F., Rosenblum, J. S., and Blobel, G. (1998) A novel nuclear import pathway for the transcription factor TFIIS. *J. Cell Biol.* 143, 1447-1455.

[98] Ferrigno, P., Posas, F., Koepp, D., Saito, H., and Silver, P. A. (1998) Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin homologs NMD5 and XPO1. *EMBO J.* 17, 5606–5614.

[99] Polizotto, R. S., and Cyert, M. S. (2001) Calcineurin-dependent nuclear import of the transcription factor Crz1p requires Nmd5p. *J. Cell Biol.* 154, 951-960.

Appendices

Appendix I-

Figures of chapter II

Fig. 3

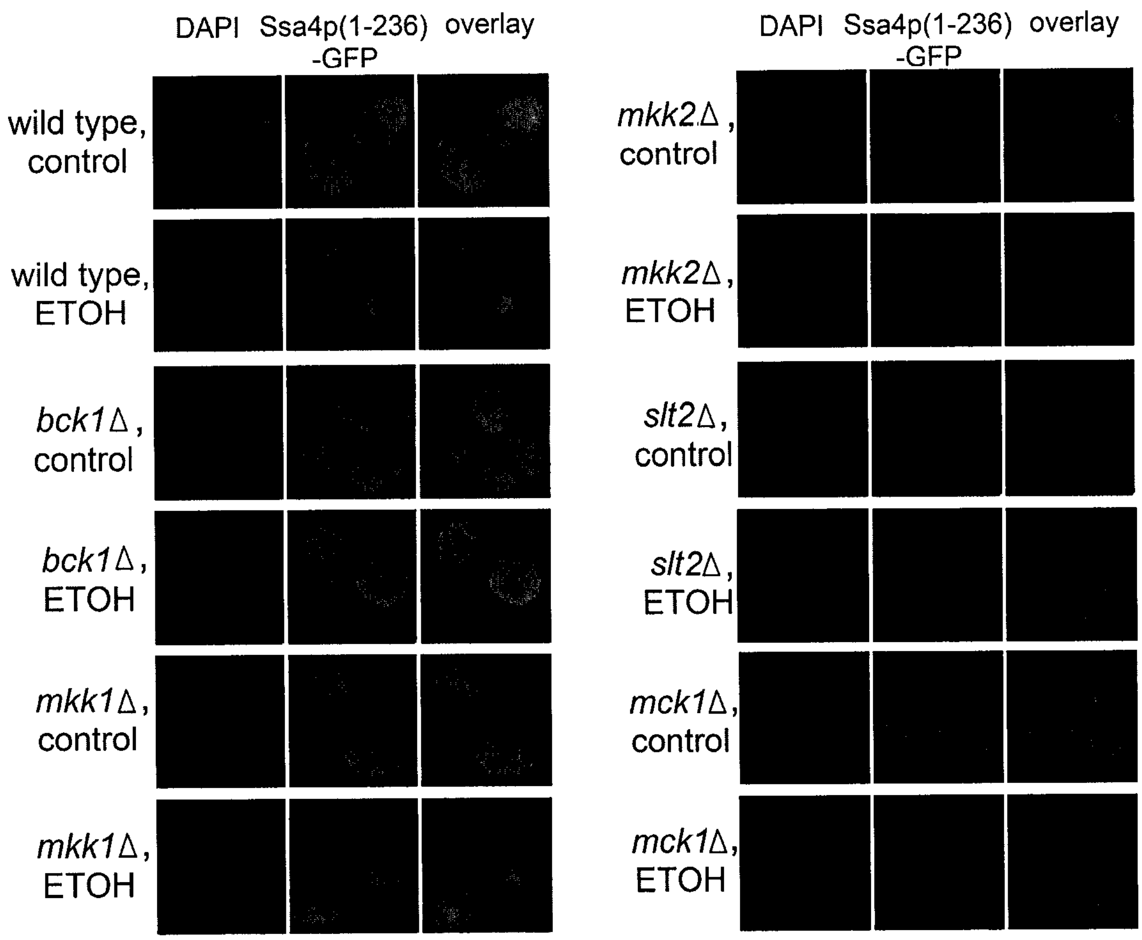

DAPI
Ssa4p(1-236) -GFP
overlay
wild type, control
wild type, ETOH
*bck1*Δ, control
*bck1*Δ, ETOH
*mkk1*Δ, control
*mkk1*Δ, ETOH
DAPI
Ssa4p(1-236) -GFP
overlay
*mkk2*Δ, control
*mkk2*Δ, ETOH
*slt2*Δ, control
*slt2*Δ, ETOH
*mck1*Δ, control
*mck1*Δ, ETOH

Fig. 3 Nuclear import of Ssa4p(1-236)-GFP upon ethanol exposure is reduced in different deletion mutants of the cell integrity MAPK cascade, but not in a deletion mutant lacking the downstream kinase Mck1p. Cell were fixed and DNA was visualized with DAPI. Ssa4p(1-236)-GFP was localized by fluorescence microscopy for wild type cells or mutants lacking the protein kinase indicated. Controls or cells incubated for 10 minutes with 10% ethanol (ETOH) are shown. Overlays of DAPI staining and green fluorescence signals are shown in the right column of each panel.

Fig. 4

A

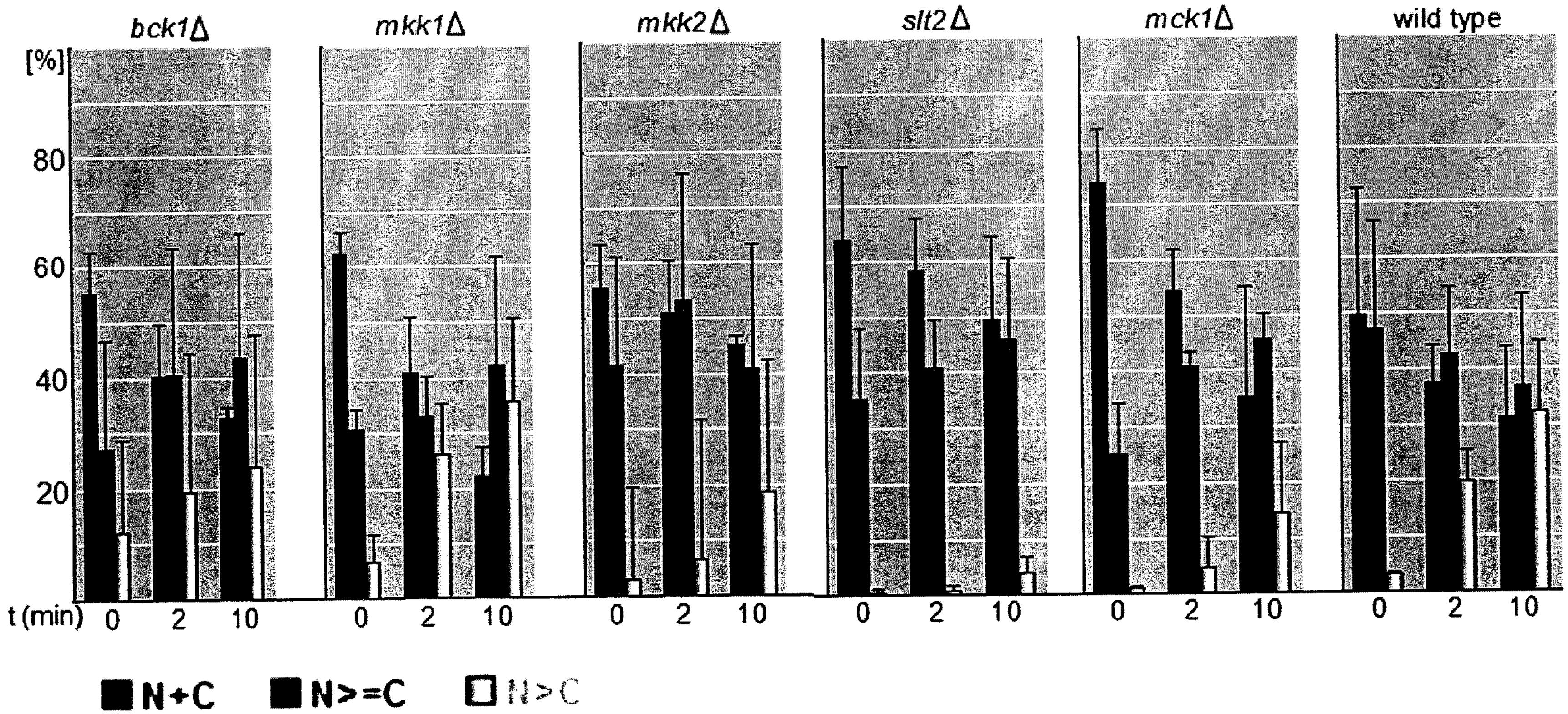

bck1Δ
mkk1Δ
mkk2Δ
slt2Δ
mck1Δ
wild type
[%]
80
60
40
20
t (min)
0
2
10
N+C
N>=C
N>C

Fig. 4

B

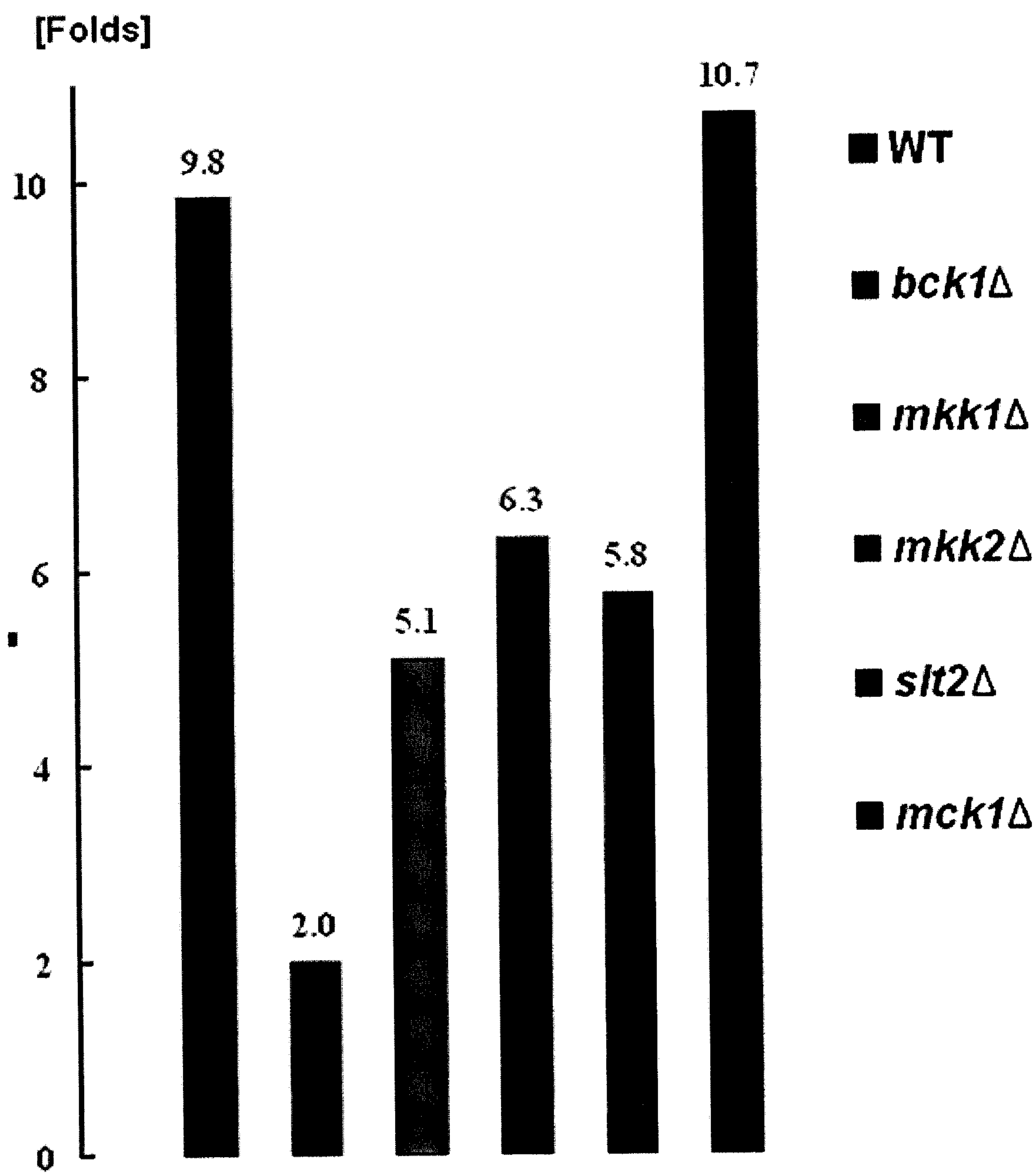
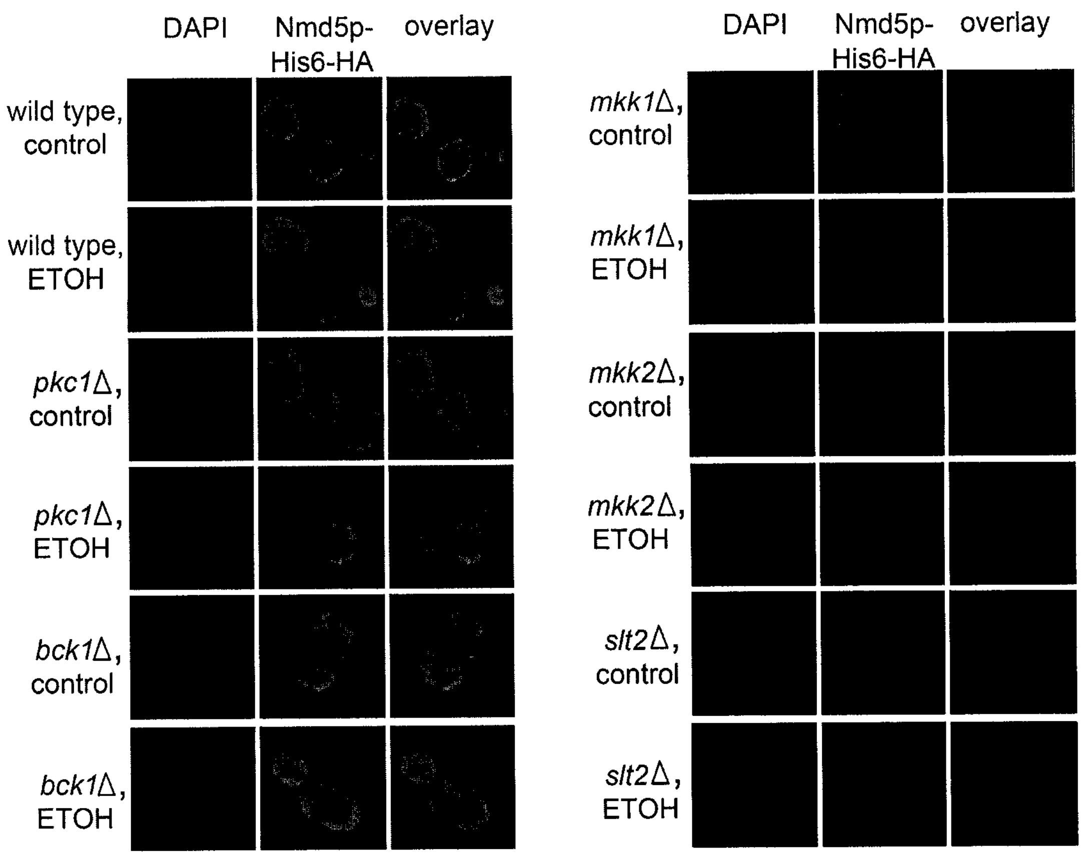

[Folds]
10
8
6
4
2
0
9.8
2.0
5.1
6.3
5.8
10.7
WT
bck1Δ
mkk1Δ
mkk2Δ
slt2Δ
mck1Δ

Fig. 4 Ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP is regulated by the cell integrity MAPK cascade.

(A) The distribution of Ssa4p(1-236)-GFP was determined for 0 min, 2 min and 10 min treatment with 10% ethanol. N+C represents the number of cells displaying similar fluorescence signals in cytoplasm and nucleus; N≥C, the number of cells with slightly stronger fluorescence signals in nuclei than in the cytoplasm; N>C, the number of cells showing much stronger fluorescence for nuclei compared with the cytoplasm. N>C is referred to as nuclear accumulation. For each set of three independent experiments at least 100 cells synthesizing Ssa4p(1-236)-GFP were examined by fluorescence microscopy. Means and standard deviation are shown.

(B) The increase of Ssa4p(1-236)-GFP nuclear accumulation was calculated using the data shown in (A) and the following formula: N>C (cells treated with ethanol for 10 min) / N>C (control, 0 min).

Fig. 5

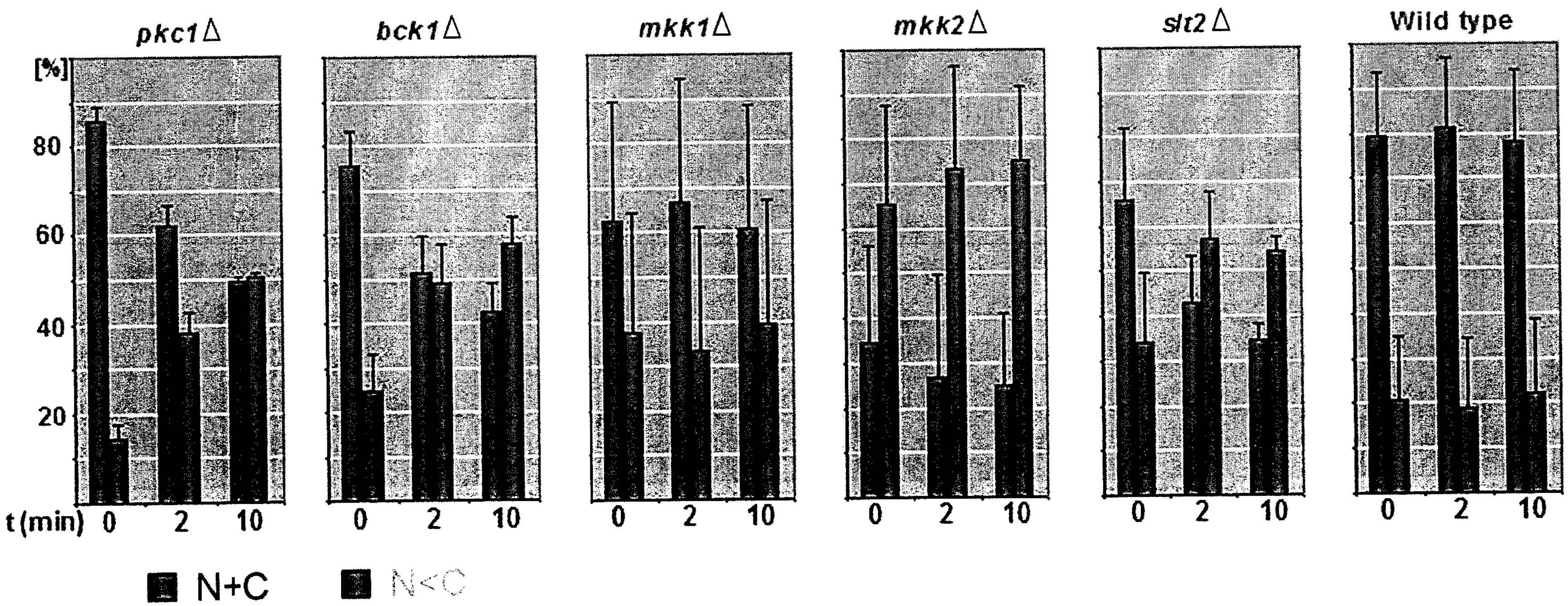


DAPI
Nmd5p-His6-HA
overlay
wild type, control
wild type, ETOH
*pkc1*Δ, control
*pkc1*Δ, ETOH
*bck1*Δ, control
*bck1*Δ, ETOH
DAPI
Nmd5p-His6-HA
overlay
*mkk1*Δ, control
*mkk1*Δ, ETOH
*mkk2*Δ, control
*mkk2*Δ, ETOH
*slt2*Δ, control
*slt2*Δ, ETOH

Fig. 5 Ethanol treatment increases the cytoplasmic localization of Nmd5p-His6-HA in *pkc1*Δ, *bck1*Δ and *slt2*Δ mutants. Wild type and mutant cells were processed as detailed in Material & Methods. Transformed cells were incubated with primary antibodies against the HA-epitope and FITC (green) or Cy3 (red) -conjugated secondary antibodies. Localization of Nmd5p-His6-HA was monitored by indirect immunofluorescence microscopy. Nuclei were visualized by DNA staining. Overlays of DAPI staining and green or red fluorescence are shown as well.

Fig. 6

A



pkc1Δ
bck1Δ
mkk1Δ
mkk2Δ
slt2Δ
Wild type
[%]
80
60
40
20
t (min)
0
2
10
N+C
N<C

Fig. 6

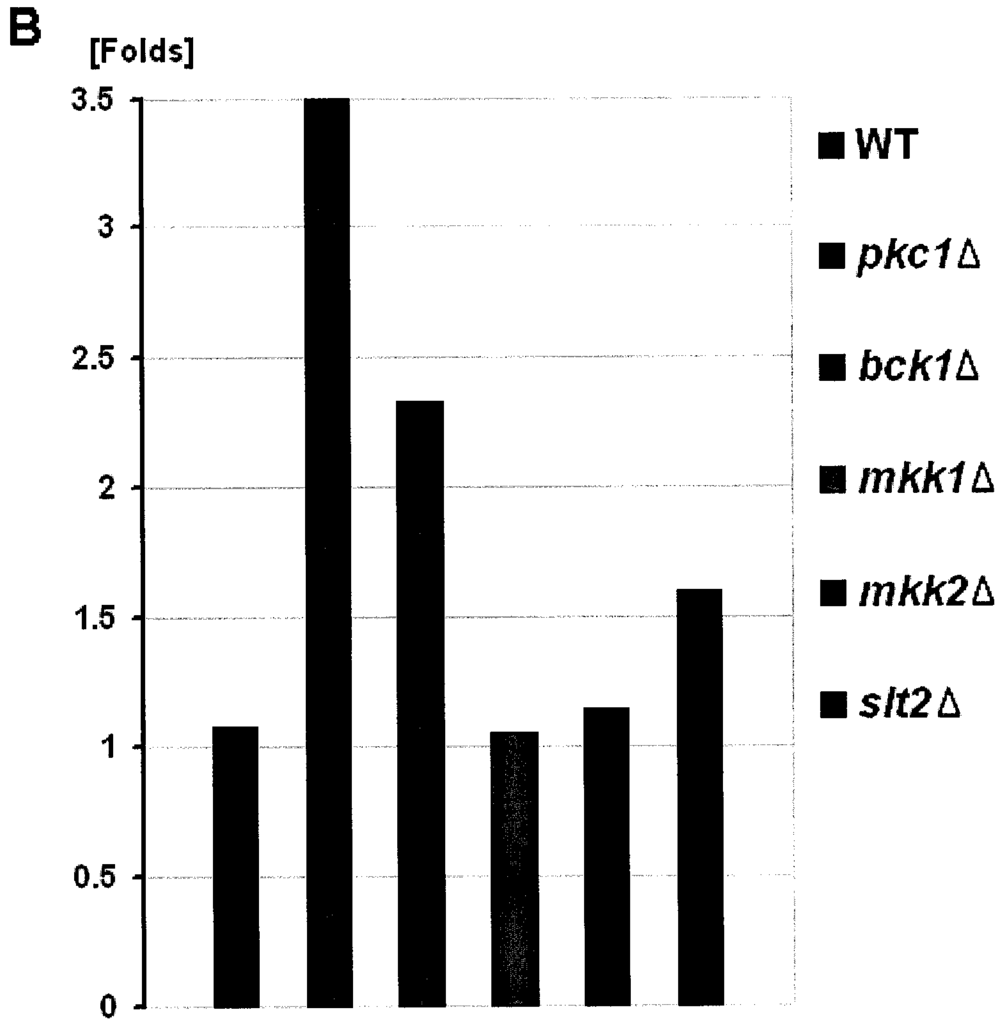


Fig. 6 Cytoplasmic localization of Nmd5p-His6-HA increases upon ethanol exposure in *pkc1*Δ, *bck1*Δ, *slt2*Δ mutants.

(A) The distribution of Nmd5p-His6-HA was quantified essentially as detailed in Fig. 4A. N<C, cells displaying much stronger fluorescence signals in the cytoplasm than in nuclei.

(B) The increase of Nmd5p-His6-HA cytoplasmic localization was calculated with the data shown in (A) and using the following formula: N<C (cells treated with ethanol for 10 min) / N<C (control, 0 min)]

Fig. 7

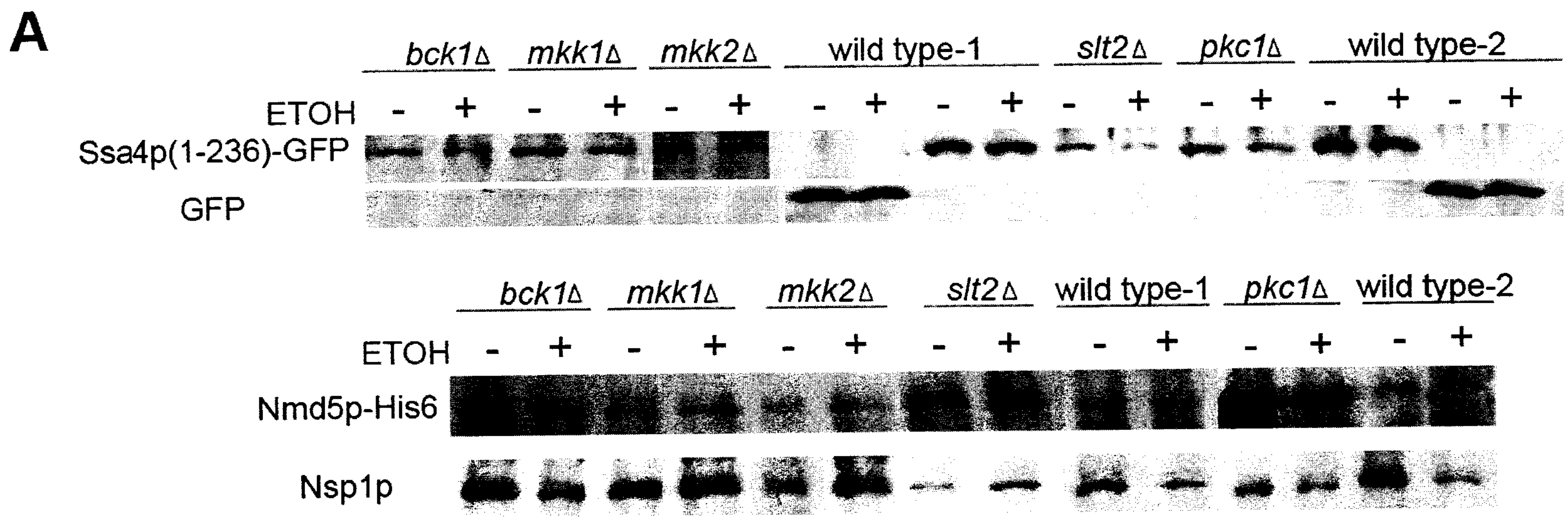

A
bck1Δ
mkk1Δ
mkk2Δ
wild type-1
slt2Δ
pkc1Δ
wild type-2
ETOH
- + - + - + - + - + - + - + - + - +
Ssa4p(1-236)-GFP
GFP
bck1Δ
mkk1Δ
mkk2Δ
slt2Δ
wild type-1
pkc1Δ
wild type-2
ETOH
- + - + - + - + - + - + - +
Nmd5p-His6
Nsp1p

Fig. 7

B

		Phosphorylated protein/total protein (%)		
		Nmd5p-His6 (%)	Ssa4p(1-236)-GFP (%)	Nsp1 (%)
Ethanol	−	16.47	22.94	29.81
		31.62	73.31	22.14
			16.77	33.05
			54.65	29.08
				52.3
				50.72
				28.31
				38.56
	+	56.16	11.19	8.53
		45.55	16.94	32.4
		38.76	76.3	26.8
		71.79	14.08	57.2
			38.76	73.63
			30	31.73
				80.69
Probability		***0.07***	***0.53***	***0.40***

Fig. 7

C

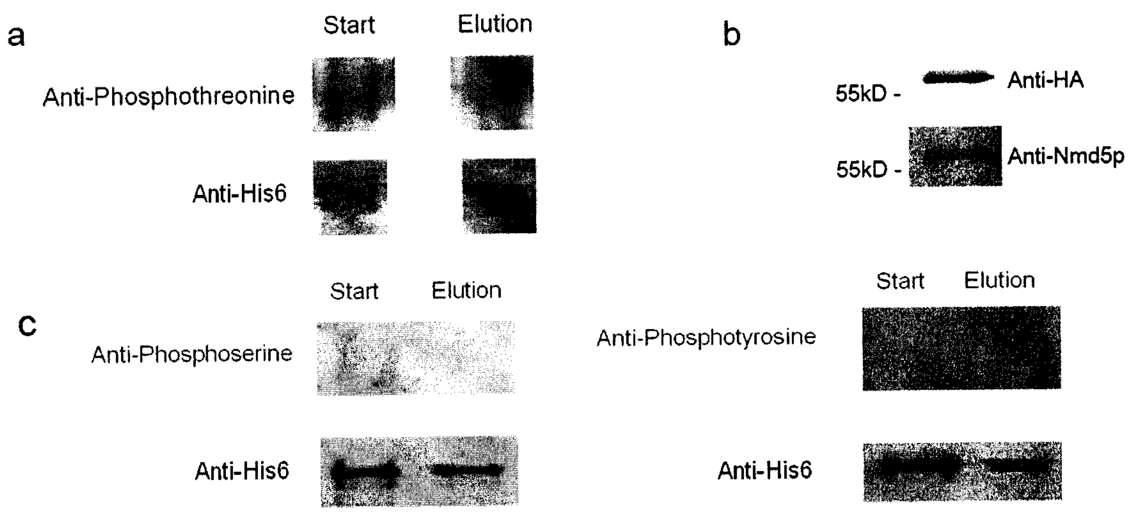

a
Start
Elution
Anti-Phosphothreonine
Anti-His6
b
55kD -
Anti-HA
55kD -
Anti-Nmd5p
c
Start
Elution
Anti-Phosphoserine
Anti-His6
Start
Elution
Anti-Phosphotyrosine
Anti-His6

Fig. 7 Phosphorylation of Nmd5p changes when cells are treated with ethanol.

(A) No change in mobility of Ssa4p(1-236)-GFP, Nmd5p-His6 and Nsp1p is detected upon ethanol stress. Wild type cells and cells carrying a deletion of one component of the cell integrity Pkc1p-MAPK cascade were stressed with 10% (v/v) ethanol for 10 minutes. Western blot analysis was carried out side by side with cell lysates from unstressed and treated cells. Antibodies against GFP were used to detect Ssa4p(1-236)-GFP and GFP. Nmd5p-His6 was located with His6-specific antibodies and Nsp1p with mAb414.

(B) The nuclear import carrier Nmd5p, its cargo Ssa4p(1-236)-GFP and the nucleoporin Nsp1p can be purified by IMAC. Nmd5p phosphorylation changed drastically in ethanol-treated cells. Phosphorylated proteins from stressed and control cell lysates were purified by IMAC and subjected to western blot analysis as detailed in Materials & Methods. An aliquot of the starting material (St; Crude cell extract), flow through (FT) and fractions eluted with sodium phosphate were separated side by side. Signals obtained after western blotting and ECL were quantified.

Relative phosphorylation (%) = [eluted protein] / [total amount of protein]

Nsp1p is potentially sensitive to proteolytic degradation and was detected as a doublet of ~90 kDa and ~80 kDa in all experiments.

(C) Nmd5p is phosphoryalted on threonine residue(s). (a, c) Nmd5p-His6 was isolated from wild type cells with Ni-NTA agarose under denaturing condition and analyzed by western blotting as described in Materials & Methods. Filters were incubated with primary antibodies against phosphoserine, phosphotyrosine or phosphothreonine and HRP-conjugated secondary antibodies. The position of Nmd5p-His6 was determined by stripping the filter and reprobing with antibodies against the His6-epitope. **(b)** Threonine-phosphorylated proteins were isolated from crude lysates by indirect immunoprecipitation with anti-phosphothreonine antibodies. Western blot analysis of isolated threonine-phosphorylated proteins was carried out and Nmd5p-His6-HA was detected with antibodies against the HA-tag. The filter was stripped and reprobed with affinity purified antibodies against Nmd5p (Anti-Nmd5p).

Fig. 8

```
UPI0000365959[Unknown]                    664 LSLAH--SLTCQQVSHQMWELLPLVYEVFQQD---GFDYFTDMMPLLHNYVTVDTDALLS 718
Q4SI91[Tetraodon nigroviridis]            665 LSLAH--SLTCQQVSPQMWQLLPLVYEVFQQD---GFDYFTDMMPLLHNYVTVDTDTLLS 719
Q6NYN3[Danio rerio]                       656 LSLAH--SLTCQQVSPQMWQLLPLIYDVFQQD---GFDYFTDMMPLLHNYITVDTDTLLS 710
O95373[Homo sapiens]                      656 FSLAH--SLTCQQVSPQMWQLLPLVFEVFQQD---GFDYFTDMMPLLHNYVTVDTDTLLS 710
O15397[Homo sapiens]                      656 LSLAY--SLTCHSISPQMWQLLGILYEVFQQD---CFEYFTDMMPLLHNYVTIDTDTLLS 710
Q7TMY7[Mus musculus]                      656 LSLAY--NLTCHTISPQMWQLLGILYEVFQQD---CFEYFTDMMPLLHNYVTVDTNALLS 710
Q5B0C4[Aspergillus nidulans FGSC A4]      675 FEIIDSCTFASKSISPTMWQAFELIHKTFKAG---AELYLEDMLPALDNYVAYGSQTLVQ 731
Q4I1J8[Gibberella zeae]                   678 FEIIDSCTFAAKSISPTMWQAFELIHTTFKAG---AEYYLEDMLPALDNFVQFGAPQLAQ 734
NMD5/YJR132W                              695 CEFVENSTF[redacted]KILELIGECNRKPDSMVSYYLSDFMLALNNILIYGRNELKK 754
P46970[Saccharomyces cerevisiae]          695 CEFVENSTFLLRDITPISWKILELIGECNRKPDSMVSYYLSDFMLALNNILIYGRNELKK 754
Q6FK77[Candida glabrata]                  694 CEFFENSTFLMRTISPIAWKVLELIGECNRREESTVSVYLEDFMLVLNNFLIYGKDELRK 753
                                                .:    .:  : ::   *: : ::     :         *: *::  *.* :  .    * .
```

Fig. 8 Nmd5p contains a sequence similar to the consensus site for Snf1p. At this site a serine/threonine residue is conserved among different species. The protein sequence of Nmd5p was used as the query for PSI-BLAST analysis against the UniRef90 protein dataset at UniProt and lined with the ten most significant hits, based on E-value, to the *S. cerevisiae* sequence [96].

Fig. 9

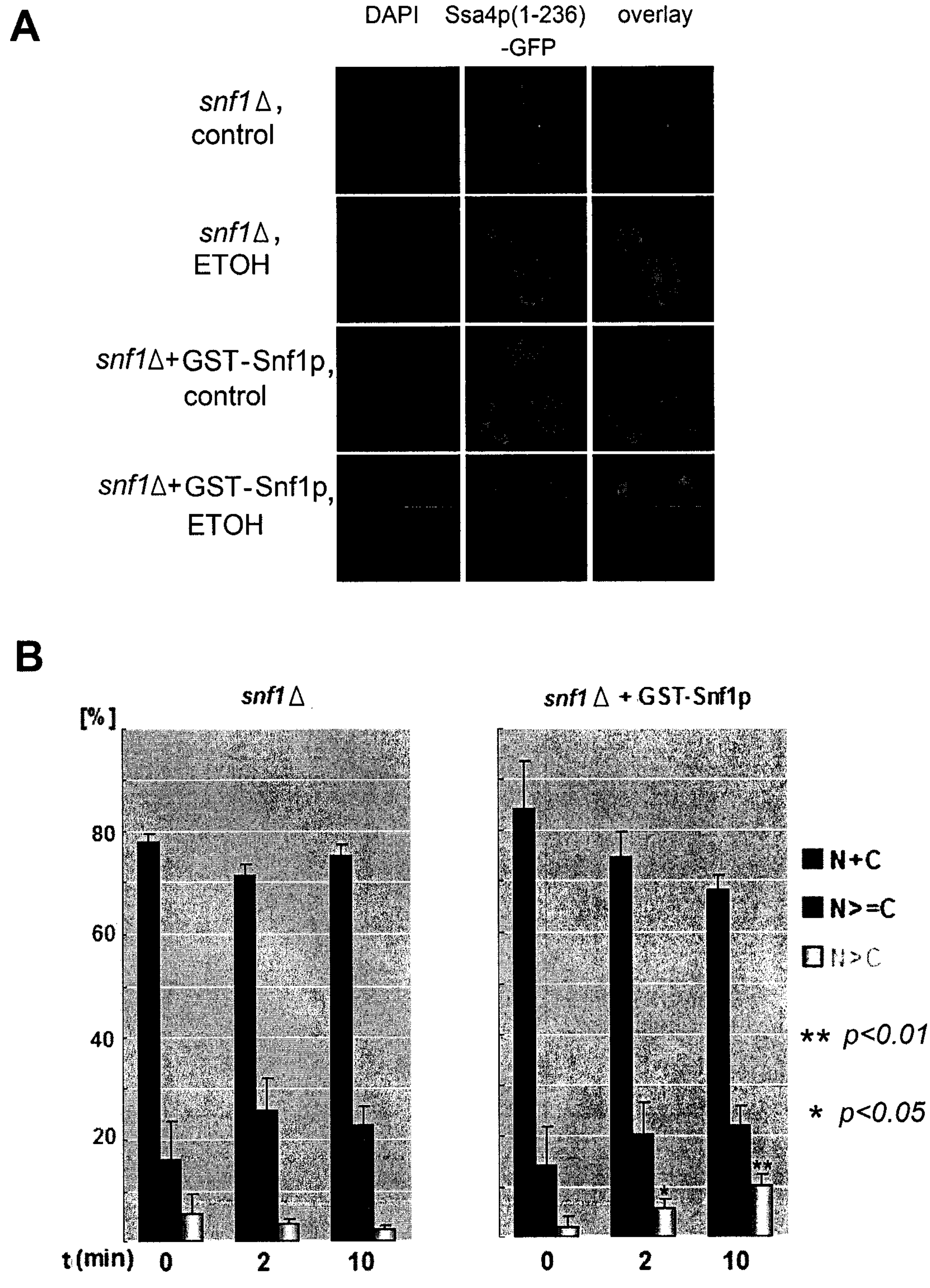

A
DAPI
Ssa4p(1-236) -GFP
overlay
snf1Δ, control
snf1Δ, ETOH
snf1Δ+GST-Snf1p, control
snf1Δ+GST-Snf1p, ETOH
B
snf1Δ
snf1Δ + GST-Snf1p
[%]
80
60
40
20
t (min)
0
2
10
N+C
N>=C
N>C
** p<0.01
* p<0.05

Fig. 9 In a *snf1Δ* mutant Ssa4p(1-236)-GFP fails to accumulate in nuclei upon ethanol stress. This phenotype can be partially rescued by overexpression of GST-Snf1p.

(A) Cells were stressed and processed for fluorescence microscopy as described for Fig. 3. *snf1Δ* mutant cells were transformed with a plasmid encoding Ssa4p(1-236)-GFP (two upper rows) or simultaneously with two plasmids encoding either Ssa4p(1-236)-GFP or GST-Snf1p (two bottom rows). DAPI stained DNA, while green fluorescence localized Ssa4p(1-236)-GFP. Overlays of DAPI staining and green fluorescence are depicted.

(B) The distribution of Ssa4p(1-236)-GFP was quantified as for Fig. 4A. Means and standard deviations are shown.

Appendix II-

Research compliance certificates