Characterization of nuclear transport pathways of heat shock protein 70 upon ethanol stress in the budding yeast *Saccharomyces cerevisiae*

by

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Table of Contents

Abstract

The main objective of this thesis is to characterize the mechanisms of nuclear transport of the cytosolic yeast heat shock protein 70 Ssa4p upon ethanol stress and the effect of stress and nutrient availability on transport carriers.

Heat shock protein 70 (Hsp70) plays an important role in cell damage recovery and transport of proteins across intracellular membranes. Upon stress, trafficking of the majority of proteins is inhibited with the exception of Hsp70 that accumulates in the nucleus. Using the budding yeast *Saccharomyces cerevisiae* as model, this thesis specifically demonstrates that Ssa4p transiently and reversibly accumulates in nuclei upon ethanol stress and exports to the cytoplasm during ethanol recovery. The cNLS-mediated classical nuclear import pathway is not required for stress-induced Ssa4p nuclear trafficking, and the N-terminal 236 amino acid residues are sufficient for Ssa4p nuclear accumulation. The importin β family member Nmd5p forms import complexes with its cargo Ssa4p and translocates cargo into the nucleus. Upon withdrawal of ethanol stress, Ssa4p exports to the cytoplasm by binding to the exporter Msn5p. Transport of Ssa4p in both directions requires the Gsp1p system. Pkc1p and the cell integrity pathway sensors are involved in Ssa4p nuclear import upon ethanol treatment.

The thesis also states that ethanol stress regulates Ssa4p nuclear transport by enhancing the import complex Ssa4p/Nmd5p formation and the docking of Nmd5p at the NPC. Stresses regulate Ssa4p nuclear export by regulating exporter

Msn5p cellular localization and formation of the export complex Ssa4p/Msn5p. Therefore, the carrier-mediated nuclear trafficking might also be regulated by the transport carrier localization. Furthermore, intracellular localization of yeast nuclear exporters is sensitive to different stresses and regulated by Snf1 kinase and cell nutrient availability.

In summary, this thesis defines the nuclear import and export mechanisms for the Hsp70 Ssa4p and their regulation in stressed cells. The novel finding of how the nutritional state and Snf1 kinase regulate exporter localization in general may provide a better understanding of protein transport regulation and the multiple levels cells use to adjust to environmental changes.

Résumé

L'objectif premier de cette thèse est de caractériser les mécanismes de transport vers le noyau de la protéine cytoplasmique de choc thermique 70 Ssa4p de la levure suivant un stress à l'éthanol, et de caractériser l'effet de ce stress et de la disponibilité des nutriments sur les effecteurs de ce transport.

La protéine de choc thermique 70 (Hsp70) joue un rôle important dans le rétablissement des dommages cellulaires et dans le transport des protéines au travers des membranes intracellulaire. Lors de stress, Hsp70 s'accumule dans le noyau alors que le transport de la majorité des autres protéines est inhibé. En utilisant la levure bourgeonnante *Saccharomyces cerevisiae* comme model, cette thèse démontre spécifiquement que Ssa4p s'accumule temporairement et de manière réversible dans le noyau à la suite d'un stress à l'éthanol, et est exportée dans le cytoplasme durant le rétablissement de ce même stress. La voie classique du transport vers le noyau médiée par le SLNc n'est pas nécessaire à l'accumulation de Ssa4p au noyau induite par le stress, et les 236 résidus d'acides aminés du côté N-terminal sont suffisants à l'accumulation de Ssa4p dans le noyau. Nmd5p, membre de la famille des importines β, forme des complexes d'importation avec son cargo Ssa4p et les déplacent dans le noyau. Lors de l'élimination du stress à l'éthanol, Ssa4p est exportée au cytoplasme en se liant à l'exporteur Msn5p du noyau. Le transport de Ssa4p dans les deux directions requiert le système Gsp1p. Durant le traitement à l'éthanol, Pkc1p et les senseurs

de la voie de l'intégrité cellulaire sont impliqués dans l'importation de Ssa4p au noyau.

Cette thèse stipule également que le stress à l'éthanol régule le transport de Ssa4p vers le noyau en augmentant la formation du complexe d'importation Ssa4p/Nmd5p et l'accostage de Nmd5p au CPN. Les stresses régulent l'exportation de Ssa4p du noyau en régulant la localisation de l'exporteur Msn5p du noyau, et la formation du complexe d'exportation Ssa4p/Msn5p. Ceci indique que les changements de localisation des effecteurs de transport pourraient être un autre niveau de régulation du trafic nucléaire médié par un porteur. De plus, la localisation intracellulaire des exporteurs du noyau de la levure est sensible à différents stresses et est régulée par la kinase Snf1 et la disponibilité en nutriments cellulaires.

En résume, cette thèse définie les mécanismes d'importation et d'exportation du noyau, ainsi que leur régulation, pour la Hsp70 Ssa4p dans les cellules stressées. La nouvelle découverte de la régulation de la localisation des exporteurs par l'état nutritionnel et la kinase Snf1 en général peu apporter une meilleure compréhension de la régulation du transport des protéines et des nombreux niveaux que les cellules emploient pour s'ajuster aux changements environnementaux.

This thesis is dedicated to my grandmother, QingLian Wu, and my grandfather, ReiChang Li, who are the first to open my eyes to the world of science, and also to my loving husband, Qiyu Qiu, who is the reason I continue to strive forward in my scientific endeavors.

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Contributions of Authors

This is a manuscript-based thesis.

Chapter 2: This paper has been published as *Quan X, Rassadi R, Rabie B, Matusiewicz N, Stochaj U. 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. 2004 May;18(7):899-901.* The full paper online is *The FASEB Journal express article 10.1096/fj.03-0947fje.* All experiments were designed and supervised by Dr. Ursula Stochaj. Rassadi R. cloned the plasmids *SSA4(1-236)-GFP* and *SSA4(16-642)-GFP* and conducted the experiments on NLS-GFP relocation upon ethanol treatment as well as all the experiments showed in Fig 2.5 after publication. Rabie B examined the association of Nmd5p-GFP with NPCs upon ethanol stress, as well as Hog1p-GFP nuclear accumulation in wild type (Fig 2.7 and Fig.2.10 after publication). Matusiewicz N purified His-tagged Nmd5p and GST-Gsp1p for the binding experiments. All other experiments were done by Quan X.

Chapter 3: This paper has been published as *Quan X, Tsoulos P, Kuritzky A, Zhang R, Stochaj U. The carrier Msn5p/Kap142p promotes nuclear export of the hsp70 Ssa4p and relocates in response to stress. Mol. Microbiol. 2006 Oct;62(2):592-609.* All experiments were designed and supervised by Dr. Ursula Stochaj. Tsoulos P. contributed to the experiments of Msn5p localization upon ethanol stress and during recovery with/without cycloheximide, as well as Mig1p-

GFP-β-gal localization upon several forms of stress and carbon sources (Fig 3.7A and 3.8 of the published paper). Kuritzky A. carried out experiments shown in Fig 3.4A and 3.6 of the publication). Zhang R. made the plasmid *SSA4(1-236)-GFP* carrying *HIS3* as a selectable marker. All other experiments were done by Quan X.

Chapter 4: This manuscript is in review: *Quan X; Yu J; Bussey H, Stochaj U. The localization of nuclear exporters of the importin-beta family is regulated by Snf1 kinase, nutrient supply and stress. BBA - Molecular Cell Research (Submit Date: Nov 23, 2006, Manuscript No.: BBAMCR-06-380).* All experiments were designed and supervised by Dr. Ursula Stochaj. Yu J. contributed to the experiments on the localization of nuclear exporters upon different stresses. Bussey H. provided Snf1 and all the MAPK deletion strains. All other experiments were done by Quan X.

List of Figures

CHAPTER 3

List of Tables

Abbreviations

ASR1	Alcohol sensitive ring/PHD finger 1 protein
BSA	Bovine serum albumin
cNLS	classical NLS
CHX	Cycloheximide
CRM1	Chromosome region maintenance
CSE1	Chromosome SEgregation
DAPI	4',6-diamidino-2-phenylindole
ECL	Enhanced chemiluminescence
EGFR	Epithelial growth factor receptor
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
EM	Electron microscopy
EtOH	Ethanol
feSEM	Field emission scanning EM
GFP	*Aequorea victoria* green fluorescent protein
GrpE	Nucleotide exchange factor for DnaK(Hsp70 in *Escherichia coli*)
Hip	Hsc70-interacting protein
Hop	Hsp70-Hsp90 organizing protein
HOG	High osmolarity glycerol response
Hsp	Heat shock protein
iFRAP	Inverse fluorescence recovery after photobleaching
JNK	c-Jun amino-terminal kinase
KAP	Karyopherin
LMB	Leptomycin B
LOS1	Loss of Suppression
MAPK	Mitogen activated protein kinase
MSN5	Multicopy suppressor of SNF1 mutation
NE	Nuclear envelope
NES	Nuclear export sequence
NLS	Nuclear localization sequence
NPC	Nuclear pore complex
Nup	Nucleoporin
PKC	Protein kinase C
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPA	Replication protein A
SAPK	Stress-activated protein kinase
SBF	SCB binding factor
SV40-NLS	NLS of SV40 T-antigen
TFIIS	Transcription elongation factor S-II
YRB1	Yeast Ran binding protien 1

CHAPTER 1

Introduction and Review of the literature

All living organisms have to cope with exposure to a variety of environmental stresses. Such stresses can originate from temperature or nutrient fluctuations, from chemical, biological or physical insults or from other forms of trauma. Living organisms have had to develop various adaptation and protection systems to enhance their probability of survival when subjected to environmental stresses.

Cells, which are the basic elements of most living organisms, are no exception. When subjected to environmental stresses, cells will respond with a variety of physiological processes aimed at preventing, minimizing or repairing damage. Over the last few decades, our basic understanding of the mechanisms involved in the response of living cells to stresses has progressed markedly. We now know that when subjected to stress, cells reprogram gene expression, produce specific proteins and modify protein transport through cell membranes. In particular, the synthesis of proteins called heat shock proteins or Hsps is enhanced when cells are subjected to stress and, in eukaryotic cells, Hsps accumulate in the nucleus, suggesting that changes occur in the transport of proteins across intracellular membranes.

The general mechanisms for the transport of proteins between the cytoplasm and the nucleus in eukaryotic cells are known and a 'classical' protein nuclear import pathway has been described in details. However, how the transport differs when cells are subjected to environmental stresses and what advantages are conferred by the relocation of proteins are still unclear.

The topic is of importance, as understanding how and why proteins are relocated when cells are subjected to stresses could potentially lead to better diagnostic and therapeutic tools for a variety of human pathologies, including viral infections, autoimmune diseases and cancer.

In this thesis we have studied the nuclear trafficking of cytoplasmic heat shock protein 70 Ssa4p in budding yeast cells subjected to ethanol stress. Before describing in more details of our research rationale and objectives, we provide below a review of the relevant literature.

1. Heat shock protein 70

In the early 1960s, it was discovered that the fruit fly *Drosophila melanogaster* had a new chromosomal puffing pattern in its salivary gland cells when exposed to temperatures higher than normal [Ritossa, 1962]. Later, it was found that these chromosomal puffs represent transcription sites for the synthesis of proteins called heat shock proteins or Hsps [Ritossa, 1962].

Since then, it has been shown that increased levels of Hsps are a universal response of living cells when subjected to a variety of stresses, including chemical (solvents, metals), physical (radiation, hypoxia, nutrient starvation, osmosis) and biological (infection, inflammation) insults [Tomasovic, 1985; Zheng, 2006]. In the case of eukaryotes, Hsps also accumulate in the nucleus in response to various stresses.

1.1 Hsp70 structure

Hsps are divided into five major families based on their molecular weight: small Hsps, Hsp60, Hsp70, Hsp90 and Hsp100. The focus of our study is the Hsp70 family.

Hsp70 proteins contain two functional domains: a 45KDa N-terminal ATPase domain and a 25KDa C-terminal substrate-binding domain [Pierpaoli, 2005] (Fig 1.1). The crystal structure of both domains has been resolved for Hsp70 in *E. coli* (DnaK) and for bovine Hsc70 [Zhu, 1996; Zhang, 2004]. The ATPase domain has two large, globular sub domains (I and II) and a cleft in between which is the nucleotide binding site that interacts with the substrate [Walter, 2002, Mayer, 2005]. The C-terminal peptide binding domain that binds to unfolded proteins is further divided into a 15KDa β-sandwich sub domain with a hydrophobic groove and a 10KDa α-helical sub domain [Mayer, 2005].

The nucleotide-bound state of the ATPase domain regulates the function of the C-terminal peptide-binding domain. In the ATP-bound state, the affinity between the substrate and the C-terminal domain is low and the exchange rate between the target substrate and Hsp70 is high. Hsp70 in the ADP-bound state has a high affinity for its substrate and a low exchange rate. These characteristics are due to the conformational change that corresponds to the ATPase cycle of Hsp70 [Walter, 2002]. When Hsp70 is in the ADP-bound state, its C-terminal α-helical sub domain covers the β-sandwich sub domain, and the substrate is trapped in the binding site. This "closed" structure has a high affinity and slow exchange rate for

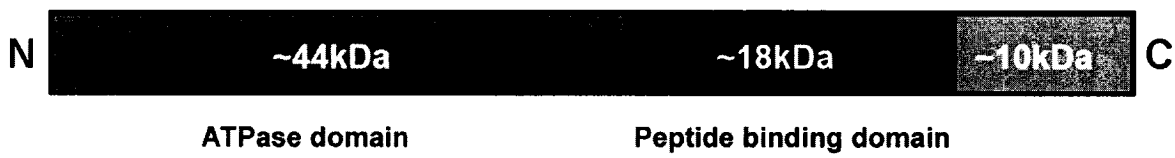

Figure 1.1 Domain structure of Hsp70 *(modified from Pierpaoli, 2005)*

the target. In the ATP-bound state, the α-helical sub domain is removed from the β-sandwich sub domain. This "open" structure has a low affinity and a high exchange rate with the substrate [Bukau, 1998; Pierpaoli, 2005] (Fig1.2).

1.2 Hsp70 Functions

Hsp70s are highly conserved and share amino acid sequences across various species, including yeast, *Drosophila*, *Escherichia coli*, *Xenopus*, mouse, chicken and man [Morimoto, 1986]. Hsp70s are found under normal conditions in various cellular compartments, including the cytosol, nuclei, mitochondria, endoplasmic reticulum (ER) and chloroplasts. Recently, it has been shown that Hsp70 can also be associated with the cell membrane [Schmitt, 2007; Lopez-Ribot, 1996].

Under normal conditions, the major role of Hsp70 located in organelles such as mitochondria or ER is to help fold and translocate nascent proteins. In bacteria, 10-20% of all proteins need Hsp70 for *de novo* folding. In eukaryotic cells, an even higher percentage of proteins depends on Hsp70 for efficient folding [Mayer, 2005].

The functions of cytosolic Hsp70s are more diverse. These proteins are involved in refolding partially denatured or misfolded proteins, preventing protein aggregation and assisting the degradation of severely damaged proteins.

Hsp70s are also required for the transport of proteins into the nucleus. *In vitro* and *in vivo* studies have shown that without Hsp70 proteins, nuclear protein

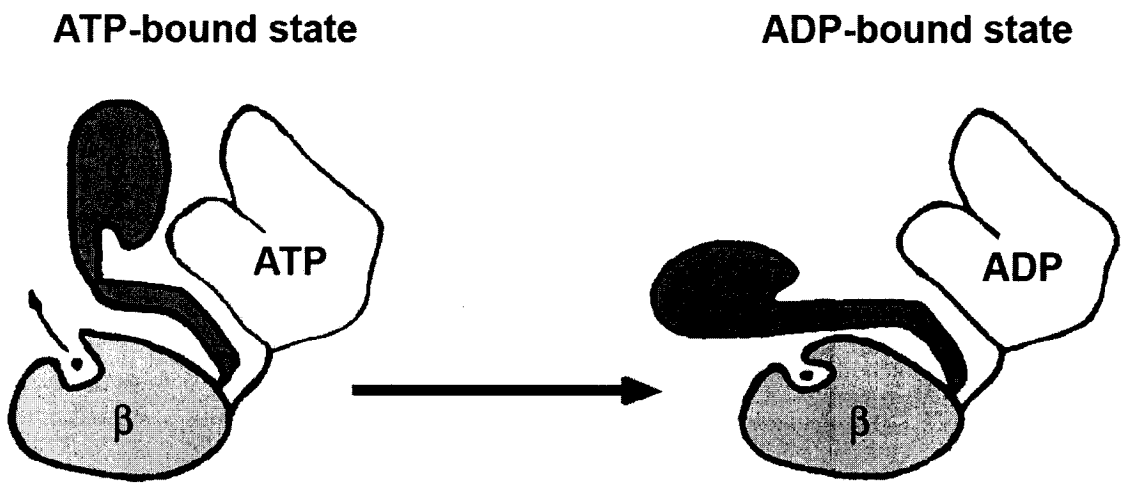

Figure 1.2 ATP- and ADP-induced conformation of Hsp70

In the ADP-bound state, C-terminal α-helical subdomain covers the β-subdomain, and the substrate (red dot) is trapped in the binding site. In the ATP-bound state, Hsp70 α-helical subdomain is removed from the β subdomain, this "open" structure has a low affinity and high exchange rate with the substrate.

(modified from Pierpaoli, 2005)

import is inhibited [Shi, 1992; Okuno, 1993]. In the S-phase, where DNA synthesis or replication occurs, there is more Hsp70 synthesized and more constitutive heat shock protein 70 (Hsc70) in the nucleus [Zeise, 1998]. Therefore, Hsp70 might play a role in regulating cell cycle by targeting specific proteins and transporting them between the nucleus and the cytoplasm. Although Hsp70s are known to be involved in shuttling proteins from the cytoplasm to the nucleus, the exact transport mechanism is still unclear.

In addition, Hsp70s interact with the components of numerous signal transduction pathways [Mayer, 2005] and play an important role in targeting proteins to organelles. As examples, mitochondrial Hsp70 (mtHsp70) is required in the mitochondrial protein import pathway [Cyr, 1997] and cytosolic Hsp70 protein is involved in regulating glucocorticoid receptor (GR) trafficking together with Hsp90 [Pratt, 2006].

The expression levels of Hsps increases markedly in response to stress and a major function of Hsp70 is to help cells and organelles recover from various forms of internal and environmental stress. Overexpression of Hsps protects cells from heat shock, ischemia, ischemia-reperfusion injury and oxidation [Zheng, 2006; Lepore, 2001]. The major mechanism seems to be the ability of Hsp70s to help stabilize and refold proteins that are partially denatured and direct severely damaged proteins to degradation through the ubiquitin pathway. Hsp70s also stabilize ribosomal subunits after heat shock by associating with them [Morimoto, 1994].

1.3 Hsp70s and pathologies

Hsp70 levels are different between normal and pathological cells. For example, Hsp70 gene expression is up-regulated by serum [Wu, 1985] and DNA virus infection [Phillips, 1991]. Many tumor cells have increased Hsp70 expression level [Soti, 2005; Multhoff, 1997]. Such overexpression of Hsp70 could be not only a potential diagnosis index, but also a therapeutic tool [Soti, 2005].

Transgenic mice with over expressed Hsp70 have increased resistance to heart ischemic damage, improved post-ischemic contractile recovery, reduced cellular damage and reduced brain ischemia injury. Hsp70 also protects skeletal muscles from damage and aid in muscle regeneration [Miyabara, 2006; Mestril, 2005]. Over expressed Hsp70 (about 350% at the protein level) can protect gastric cells from apoptosis and necrosis by the cytotoxic oxidant ammonium chloride induced [Oyake, 2006]. The inducible Hsp70s in the small intestine and colon are also important to maintain mucosa integrity and protect the cells from oxidant injury [Petrof, 2004].

In infectious diseases, a pathogen's own elevated synthesis of Hsp70 is essential for its survival inside the host, which triggers the immune system to recognize Hsp70s as antigens, inducing strong hormonal and cellular immune responses [Zugel & Kaufmann, 1999]. In several autoimmune disorders, Hsp70 levels are increased and Hsp70s are present on the cell surface. Increased expression of microbial Hsp70s could lead to a change of the host's immune

response to Hsp70s. The host could overexpress *HSP70s* upon the stress triggered by infection. Both bacterial and endogenous Hsp70s would activate a specific immune reaction against the host's own Hsp70s. This could be the mechanism responsible for autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease.

In normal cells, Hsp70 is located in both the cytoplasm and the nucleus, whereas in cancer cells Hsp70s are also localized on the cell membrane [Radons, 2005]. These Hsp70s at the cell membrane can be recognized by the immune system and trigger anti-cancer responses [Radons, 2005]. This could potentially lead to Hsp70-related anti-tumor vaccination for cancer immunotherapy [Soti, 2005; Chu, 2000].

1.4 Cytosolic Hsp70 families in yeast *Saccharomyces cerevisiae*

Numerous studies have shown that yeast is a good model system for the study of many human diseases. Many important basic cellular functions, such as protein transport, replication and recombination, and cell division are conserved between yeast and mammals. Furthermore, the yeast genome is completely sequenced. These factors make *S. cerevisiae* a good model organism to study Hsp70 protein transport at the molecular level.

Several groups of Hsp70 have been identified in the budding yeast *S. cerevisiae*. These include the *SSA* and *SSB* groups located in the cytoplasm, Kar2p (also called *SSD1*, GRP78 or BiP) in the ER and the SSC proteins located in mitochondria [Morimoto, 1994; Lopez-Ribot, 1996].

The cytosolic Hsp70 *SSA* and *SSB* groups have four members (*SSA1*, *SSA2*, *SSA3* and *SSA4)* and two members (*SSB1* and *SSB2)*, respectively [Boorstein, 1994]. The four *SSA* subfamily members are similar to each other: *SSA1* and *SSA2* are 97% identical whereas *SSA3* and *SSA4* are about 85% identical [Morimoto, 1994].

The *SSA*, but not the *SSB* subfamily, is essential for yeast cell viability and at least one Ssa protein must be synthesized at a high level [Morimoto, 1994]. Upon stress the expression levels of *SSA1*, *SSA3* and *SSA4* dramatically increase whereas that of *SSA2* remains the same. *SSA3* is induced upon starvation or under low intercellular cAMP levels, whereas *SSA4* is induced upon heat shock treatment [Boorstein, 1990]. In contrast, *SSB1* and *SSB2* genes have lower expression levels upon heat shock compared to their moderately high level expression under normal conditions.

Ssa4p is the classical Hsp70 among the yeast cytosolic Hsp70 members. It has a low expression level under normal growth conditions and a high induction upon heat shock [Boorstein, 1990]. *SSA4* has been conserved amongst closely related yeasts separated by hundreds of millions of years of evolution, implying an important function. So far, few reports characterize Ssa4p and its functions in protein folding and transport.

2. Nucleocytoplasmic transport

The main difference between eukaryotic and prokaryotic cells is the separation of nucleoplasm and cytoplasm. In eukaryotic cells, these two

compartments are separated by a nuclear envelope (NE) consisting of two lipid bilayer membranes. The outer nuclear membrane extends into the endoplasmic reticulum (ER). Embedded between the double membranes of the NE, the nuclear pore complexes (NPCs) provide the only path that allows transport of macromolecules in and out of the nucleus.

Trafficking between nucleus and cytoplasm through the NPC is a fundamental and essential cellular function. It is required for cell growth, gene expression, nuclear function, protein synthesis, signal transduction and response to stress [Gorski, 2006]. In growing cells, many molecules such as transcription factors, histones and snRNPs need to be imported from the cytosol to the nucleus. Molecules such as mRNA, tRNA and ribosomal proteins need to be exported from the nucleus to the cytoplasm for protein translation.

Ions, some RNAs and proteins smaller than 20–40 kDa in size, transport through the NPC by diffusion. However, larger proteins, RNAs and RNPs pass through NPCs using energy-dependent, signal and carrier-mediated active transport pathways [Gorlich & Kutay, 1999].

2.1 Nuclear pore complex

In yeast, there are about 200 NPCs per cell [Rout, 1993], often gathered in clusters of 3-20 [Kiseleva, 2004]. In mammalian cell there are 3000-5000 NPCs [Cordes, 1995]. Approximately 500-1000 macromolecules are transported through one NPC every second [Suntharalingam, 2003].

2.1.1 NPC structure

NPCs are composed of approximately ~30 different proteins (nucleoporins, Nups) and their size is about 60Mda in yeast [Kiseleva, 2004]. The general structure and components of NPCs are conserved amongst different species [Suntharalingam, 2003]. For example, comparing yeast with vertebrates, about 67% nucleoporins have similar structure, location and function [Suntharalingam, 2003]. NPCs are also characterized by an eightfold rotational symmetry. On the outer nuclear membrane, NPC filaments extend from the thin rings, called cytoplasmic rings, embedded in the NE. On the inner nuclear membrane, thin rings similar to cytoplasmic rings are called nuclear rings. The eight filaments extending from nuclear rings form a basket-like structure on the nucleoplasmic side which is called basket ring. Between cytoplasmic rings and nuclear rings, the encircled eight spoke rings form a central region or channel which allows the particles to translocate through NPCs. The length of an NPC is about 120nm [Pante, 2004].

The three dimensional structure of yeast NPCs has been described in detail using scanning electron microscopy. Yeast NPCs are ~95nm in diameter and have a large cytoplasmic ring which has eight subunits above the outer NE [Kiseleva, 2004]. They also have cytoplasmic filaments and nuclear baskets similar to vertebrates [Kiseleva, 2004]. A central plug or central particle is often observed in the central channel of yeast NPCs. This structure is believed to be transporters or import/export complexes going through the NPC rather than components of the NPC [Beck, 2004; Stoffler, 2003].

2.1.2 Yeast nucleoporins

In the budding yeast *S. cerevisiae,* the NPC is a large macromolecular structure containing about 30 different Nups [Shulga, 2000; Tran, 2006]. Nups are generally conserved across different species. Furthermore, between vertebrates and yeast, Nups near the pore membrane of the NPC have similar functions, although their gene sequences and protein structures are different [Suntharalingam, 2003]. Nups are classified into several groups: transmembrane proteins, FG-Nups (phenylalaine-glycine repeats), non-FG-Nups. The largest group of yeast Nups is the FG-Nups which represents more than one third of the total Nups [Suntharalingam, 2003].

Some FG-Nups are found only on side of the NPC, except symmetrically located FG-Nups which are found on both sides of the NPC. For example, Nup1p, Nup2p and Nup60p are restricted to the nuclear side, while Nup82p, Nup159p and Nup42p are only on the cytoplasmic side [Rout, 2000]. Ten out of the total yeast Nup genes are essential for cell survival under any growth condition and only six of the Nup gene mutations cause temperature sensitive lethality [Suntharalingam, 2003].

So far, there are several Nup subcomplexes identified in budding yeast which are necessary for NPC assembly. For example, the Nup84p complex, which includes Nup85p, Nup120p, Nup145C and Nup84p, is a self-assembled Y-shaped structure and is involved in polyA+ RNA export. Mutation of any gene of the Nup84 complex can result in constitutive clustering of NPCs [Suntharalingam,

2003]. The Nsp1p complex is composed of three FG-Nups, Nsp1p/Nup49p/Nup57p, and binds to Nic96p located on the NPC center core. This complex plays a role in nuclear import through the FG repeat domain.

2.1.3 The role of nucleoporins in nuclear transport

Four steps are needed for nucleocytoplasmic transport through the NPC. The first step is for a cargo protein to be recognized by a transport receptor. The cargo binds to a transport receptor (an importin β family member), through either a nuclear localization sequence signal (NLS) for import or a nuclear export signal (NES) for export, and forms an import/export complex.

The second step of transport is docking of the complex to the cytoplasmic side of the NPC, that is, the filaments. In general, the transport receptor importin β interacts with the FG repeats domain of a nucleoporin, whereby each FG-Nup can bind to a number of Kap proteins, mostly through their phenylalaine residues. Nup116p and Nup100p bind directly to the classical importin protein Kap95p during nuclear protein import. Nup53p which is located at multiple sites of NPCs, colocalizes with Nic96p, and interacts with Kap95p to play a direct role in NLS-mediated protein import [Fahrenkrog, 2000].

The third step of transport is the passage of the import complex through filaments and across NPCs. Presently, three different models are used to describe this passage [Suntharalingam, 2003]. In the Brownian model, when passive diffusion is inhibited by the FG-rich filaments, the interactions of transport complexes with FG-Nups increase their local concentration and thus increase the

chance of the complex to enter and pass through NPC. In the selective phase model, FG-Nups interact weakly with each other by their FG domains and form a hydrophobic network. The association with Kap-cargo transport complexes can break this hydrophobic network resulting in transport across the NPCs. The third model is the affinity gradient model, which proposes that FG-Nups provide a series of binding sites with increasing affinity from one side of the NPC to the other. The complex which binds to the FG-Nups has a greater possibility of transporting through the NPC while the nonbinding molecules cannot follow the affinity gradient and thus are excluded from transport [Suntharalingam, 2003].

Once the transport complex passes to the nuclear side of the NPC, the fourth and final step of transport is disassembly. On the nuclear side, a high concentration of GTP-bound Ran/Gsp1p interacts with Kaps, destabilizing the transport complex and releasing its cargo from the transport receptor [Stochaj, 1999].

Nups on the nuclear basket of NPC have important functions for both import and export. For example, Nup1p, located only at the nuclear basket of NPCs, serves as the final release site for the nuclear import complex and as the docking site for the nuclear export complex. Another nuclear side protein of the NPC, Nup2p, aids both in Kap-cargo nuclear import and nuclear export of Kap60p/importin-α [Matsuura, 2003].

2.2 Transport signals

Bidirectional transport of most macromolecules through the NPC requires a transport signal in addition to a carrier and energy. The Classical nuclear import depends on an import signal called the classical nuclear localization sequence (cNLS) and the protein exiting the nucleus normally needs a nuclear export signal (NES).

Various types of NLSs have been discovered so far. The first NLS was found in SV40 large T antigen. It is a monopartite NLS with a short piece of basic amino acids sequence of P**KKKRK**V. A bipartite NLS consisting of two short stretches of basic amino acids sequences separated by a linker of 10-12 amino acids, such as the nucleoplasmin NLS **KR**PAAIKKAGQA**KKK** as also been found. Other types of NLS are different from the classical NLS. The identification of import signals which differ from the classical NLS is an area of active research.

Generally, NESs are variations of leucine-rich peptides. Viral and vertebrate NESs have conserved hydrophobic residues separated by regular space as Φ-x_{2-3}-Φ-x_{2-3}-Φ-x-Φ (Φ=L,I,V,F,M; x is any amino acid), which are important for signal function [Kutay, 2005]. Importin β family member Xpo1p/Crm1p binds to the NES and forms an export complex in the presence of GTP-bound Ran/Gsp1p. After crossing the NPC, the complex dissociates at the NPC cytoplasmic filament and releases the NES cargo to the cytoplasm [Kutay, 2005]. It has been found that more than 75 proteins have a NES signal and can be

exported by Crm1p, such as viral HIV Rev protein and the protein kinase A inhibitor.

Proteins lacking a leucine-rich NES signal require different carriers for the nuclear export instead of Crm1p. For example, importin β family member Msn5p is able to export phosphorylated protein Pho4p, Cse1p recognizes importin α and Los1p is required for tRNA nuclear export. In mammalian cells, Crm1p is the target for the antifungal drug leptomycin B (LMB). Therefore LMB inhibits NES-mediated nuclear export. In budding yeast Xpo1p, the homolog of Crm1p, is not sensitive to LMB.

2.3 Ran/Gsp1p system

The major energy source required for nucleocytoplasmic trafficking comes from the Ran/Gsp1p system. Ran, a homolog of yeast Gsp1p, is a small GTPase belonging to the Ras superfamily containing a guanine nucleotide binding domain. Ran/Gsp1p is predominantly located in the nucleus with small amounts in the cytoplasm. Ran/Gsp1p has two states: a GTP-bound state and a GDP-bound state. The high concentration gradient of RanGTP:RanGDP is the key factor regulating the direction of nucleocytoplasmic transport. In the nuclear compartment, the import complex requires GTP-Ran/Gsp1p to interact with importin and the unstable complex releases the cargo in the nucleus; importin and RanGTP form a complex and return to the cytoplasm where GTP hydrolysis ends the cycle and releases the carrier. The free importin is then able to undergo another round of transport and the cycle is repeated. The formation of the export

complex requires the presence of GTP-Ran/Gsp1p in the nucleus; once transported to the cytoplasm, GTP hydrolysis dissociates the complex (Fig 1.3) [Weis, 2003; Pemberton, 2005].

The intrinsic cycle of Ran/Gsp1p is regulated by several modulating proteins. GTP-Ran/Gsp1p is mostly located in the nucleus while GDP-Ran/Gsp1p is in the cytoplasm. On the nuclear side, the guanine nucleotide exchange factor (RanGEF; Prp20p in *S. cerevisiae*) changes the GDP-bound state of Ran/Gsp1p to the GTP-bound state. On the cytoplasmic side, the Ran/Gsp1p GTPase activating protein (RanGAP; Rna1p in *S. cerevisiae*) stimulates GTP hydrolysis in the presence of accessory proteins RanBP1 (Yrb1p in *S. cerevisiae*) and RanBP2, and converts GTP-bound Ran/Gsp1p to GDP-bound Ran/Gsp1p. To maintain the high concentration gradient of nuclear Ran/Gsp1p, nuclear transport factor 2 (Ntf2) transports GDP-Ran/Gsp1p from the cytoplasm back to the nucleus.

2.4 Imp-β mediated transport pathway

Karyopherins (Kaps), also called Importin β, are the largest and best known soluble transport carriers which can bind to cargo proteins with a specific import or export signal (NLS or NES) and direct cargos to dock at the NPC. In *S. cerevisiae*, there are fourteen members of the importin β family and at least 20 members have been described in mammalian cells. Based on their transport direction, proteins in yeast can be classified into ten importins, three exportins and one importin/exportin. Studies have shown that each transport receptor may have

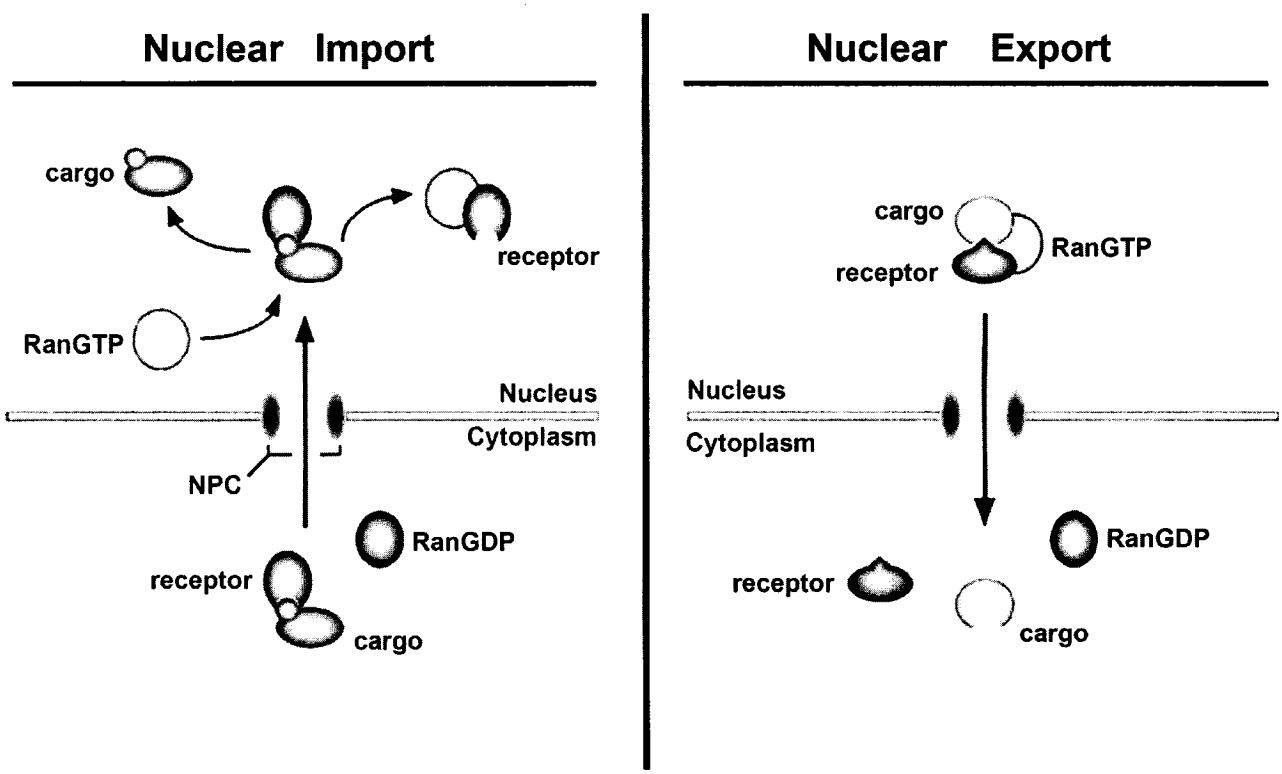

Figure1.3 RanGTPase cycle and nuclear import/export.

(modified from Nakielny and Dreyfuss, 1999)

Figure 1.3 The Differential Effects of RanGTP on Nuclear Import and Nuclear Export Receptor-Cargo Complexes.

Import receptors bind their cargos in a RanGTP-independent manner and RanGTP causes dissociation of these complexes. They are thus permitted to form in the cytoplasm and dissociate in the RanGTP-rich nucleus. Export receptors form stable complexes with their cargos only in the presence of RanGTP. These ternary complexes are thought to be the export unit, and dissociate in the cytoplasm and/or on the cytoplasmic filaments of the pore where RanGAP activity converts the RanGTP to RanGDP.

more than one cargo binding site and the ability to mediate multiple protein transport under various conditions [Pemberton, 2005; Chook, 2001].

2.4.1 Importins

The molecular weights of the importin β proteins are in the range of 95-145KDa. Only 20% of their gene sequence has been identified. Their basic structure is similar and composed of approximately 20 HEAT repeats. Each HEAT repeat is about 40 amino acid residues in length and consists of two helices with a short loop in between. The 20 HEAT repeats, a super helical structure with arches in both N- and C-terminal regions, form an extensive interaction surface to favor the association with more than one protein at the same time. HEAT is an abbreviation of Huntington motif, Elongation factor 3, A subunit of protein phosphatase 2A and TOR1 [Harel, 2004].

In the N-terminal region of importin β, the Ran-binding domain occupies HEAT repeats 1-8. The central part is the nucleoporin-binding domain located at HEAT repeats 4-8, and the C-terminal cargo-binding domain is at repeats 7 to 19. At repeat 8 there is an acidic loop instead of a HEAT repeat, which has an important role in regulating the cargo interaction or release by interacting with Ran/Gsp1p (Fig 1.4) [Strom, 2001]. Importin β has the ability to bind cargos directly or through adaptor proteins such as importin α (Kap60p; Srp1p in *S. cerevisiae*).

Among the ten importin members in the budding yeast, Kap95p (Importin β1 in mammalian cell) is the classical and well defined importin β protein. It

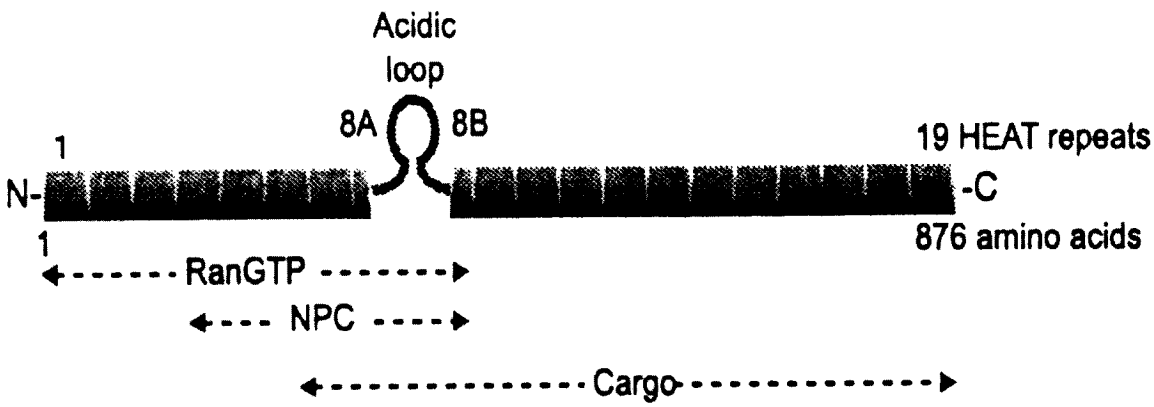

Figure 1.4 Importin β structure.
Importin β is composed of 19 HEAT repeats. in HEAT-8 is replaced by an acidic loop critical for the regulation of substrate binding and release.

(modified from A. Ström, 2001)

recognizes many cargos with classical NLS (cNLS) through the adaptor importin α (Kap60p). The Kap95p-Kap60p-cNLS-cargo forms an import complex and binds to the FG-Nup Nsp1p of the NPC filament, followed by transport through the NPC. At the nuclear side of the NPC, GTP-bound Ran/Gsp1p binds with high affinity to Kap95p probably by interacting with both N-terminal HEAT repeats and the acidic loop of Kap95p [Chook, 2001]. The association of Kap95p and GTP-Ran/Gsp1p destabilizes the import complex and releases the cargo in the nucleus. This classical import pathway requires several key factors: transport signal cNLS, adaptor importin α/Srp1p, transport receptor importin β/Rsl1p and the Ran/Gsp1p system (Fig 1.5).

Several importin β members, including Kap123p, Kap108p/Sxm1p, Kap121p/Pse1p and Kap95p, are involved in mediating nuclear import of ribosomal proteins. Kap104p is important in mediating a nuclear import of mRNA-binding proteins and Kap114p imports TATA-binding proteins [Ström, 2001]. Nuclear accumulation of cytoplasmic tRNA requires Kap111p/Mtr10p together with Ran [Shaheen HH, 2005].

2.4.2 Exportins

There are three importin β family members identified as nuclear exportins. Msn5p functions both as importin and exportin. Xpo1p, the yeast homolog of Crm1p, recognizes leucine-rich NES signals and exports NES-containing proteins, such as the mitogen-activated protein kinase Hog1p, Hsp70 family member Ssb1p and the Snf1 kinase ß subunit Gal83p. The Crm1p-dependent

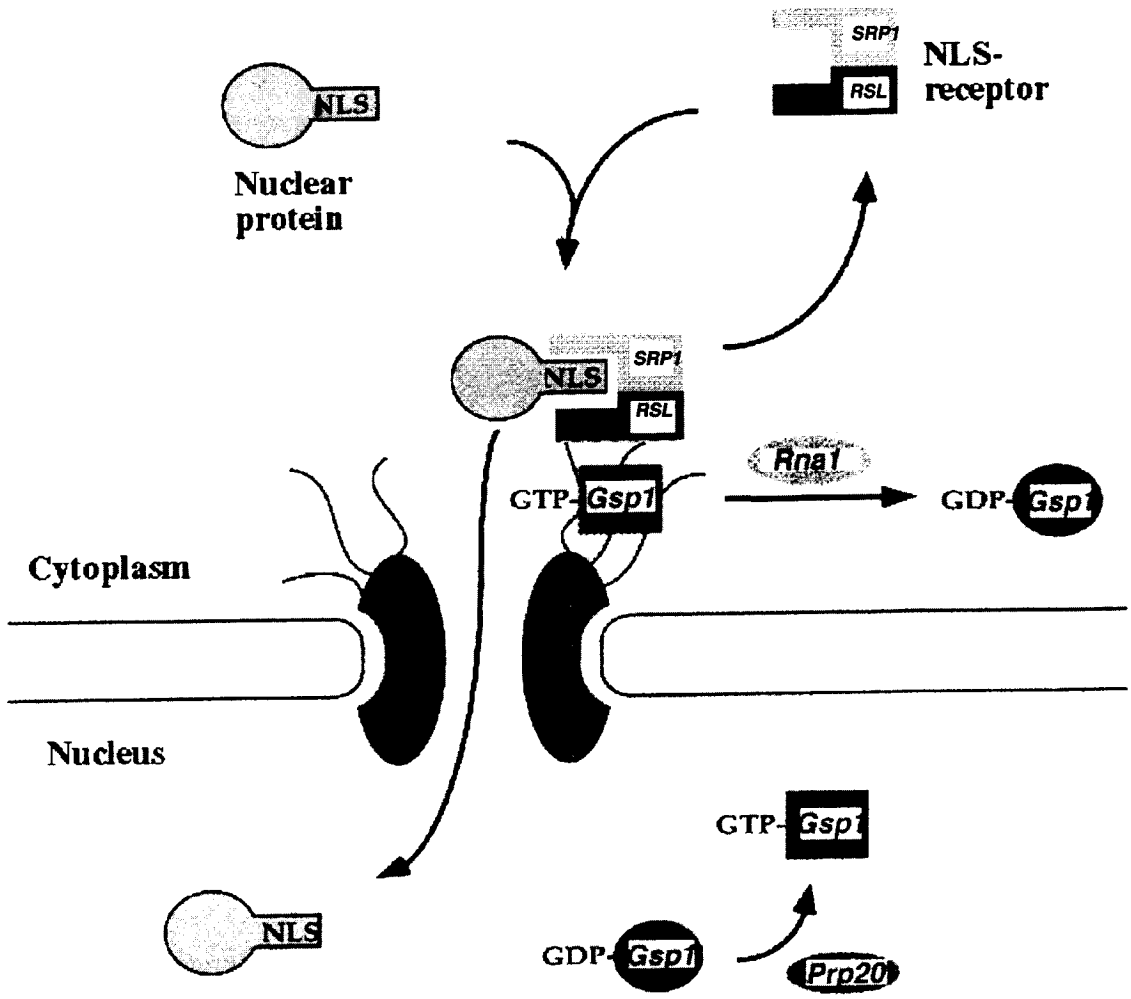

Figure1.5 cNLS-mediated classical import pathway.

(modified from U. Stochaj)

nuclear export is important for the cytoplasmic retention of Gal83p in glucose-grown cells [Hedbacker, 2006]. Xpo1p is also involved in mRNA and ribosomal protein nuclear export. Ltv1p, a nonribosomal protein that is required for ribosomal 40S subunit synthesis in yeast, contains a functional NES and exports from the nucleus in a Crm1p-mediated manner [Seiser, 2006]. Besides the nuclear transport function, Crm1p also plays a role in cell cycle progression. In the S phase, in the presence of RanGTP, Crm1p binds to the MCM complex (one of the pre-replicative complexes) and inhibits DNA re-replication [Mosammaparast, 2004].

Cse1p (mammalian CAS) together with RanGTP, interacts with Srp1p/importin α and plays an important role in importin α nuclear export and recycling. After importin β/importin α/cargo complex imports into the nucleus, the receptor and adaptor return to the cytoplasm for the next transport cycle. Importin α requires its carrier Cse1p and RanGTP to form a nuclear export complex in order to translocate back to the cytoplasm. This complex is disassembled upon RanGTP hydrolysis to RanGDP in the cytoplasm. Cse1p HEAT motifs have a Ran-binding site and cargo binding sites forming a ring-like structure. In the absence of cargo, the Ran-binding site is covered and the cargo binding site is twisted thus preventing the cargo-free form of Cse1p from binding to substrate proteins lacking RanGTP in the cytoplasm. In the nucleus, the Cse1p structure is changed into an open state and allows RanGTP and cargo interaction [Cook, 2005]. Recently it has been reported that Cse1p is also a chromatin-

associated protein in yeast and plays a role in gene regulation and DNA replication [Yu, 2006].

Los1p (exportin-t in mammalian cells) is another exportin which mediates tRNA nuclear export [Abruzzi, 2007]. Msn5p/Kap142p has various functions as transport receptor. As a transport receptor, Msn5p can mediate nuclear import of some proteins and also mediate nuclear export of different proteins [Quan, 2006]. Msn5p exports cargos that are phosphorylated. Transcription factor Pho4p, which is regulated in response to changes in phosphate availability, must be phosphorylated for its interaction with RanGTP and Msn5p and export from the nucleus [Kaffman, 1998]. Another transcription factor involved in cell cycle regulation, Swi6p, also requires Msn5p as its nuclear export carrier, confirming Msn5p's role in cell cycle control [Queralt, 2003]. The cyclin-dependent kinase inhibitor Far1p mediates cell cycle arrest in the nucleus. Nuclear Far1p relocates to the cytoplasm in response to pheromones, which requires Msn5p as its exporter in the presence of GTP-bound Gsp1p [Blondel M, 1999]. Msn5p, as yeast importin, mediates nuclear import of replication protein A (RPA) which plays an important role in DNA metabolism including DNA replication, DNA repair and recombination. Msn5p binds to RPA and forms a nuclear import complex which can be dissociated by RanGTP [Yoshida, 2001].

3. Kinase in protein nuclear transport regualtion

3.1 PKC-cell integrity and MAPK pathway

Protein kinase C (PKC) is a serine/threonine protein kinase conserved in eukaryotes and essential for controlling cell wall integrity signaling. In mammalian cells, at least 10 isoforms of PKC have been identified, but in yeast *S. cerevisiae* the only isozyme present is Pkc1p [Denis, 2005]. Pkc1p is required for normal growth and division of yeast cells.

The cell integrity pathway is essential to maintain the wall shape and integrity of yeast cells and consists of a series of phosphorylation reactions triggered by activated Pkc1p. In yeast *S. cerevisiae* Pkc1p is activated by Rho GTPase. Pkc1p then activates a linear mitogen-activated protein kinase pathway (MAPK), the main downstream target of Pkc1p. The MAPK cascades comprise the MEK-kinase Bck1p, a pair of redundant MEK-kinases Mkk1p and Mkk2p, and the MAP kinase Slt2p. One of the downstream signals activated by MAPK cascades is the serum response factor-like transcription factor Rlm1p. Rlm1p regulates expression of genes involved in cell wall biogenesis.

MAPK cascades are not the only downstream target of Pkc1p. There are several other signaling events activated through Pkc1p, such as actin depolarization, transient nuclear protein export upon hypertonic shock and N-glycosylation activity. Bck2p, which activates cell cycle related transcription factor SBF in the nucleus, is another effecter of Pkc1p, [Perez, 2002].

There are five MAPK pathways in yeast. The Fus3 MAPKs regulate mating in response to pheromone; the Kss1 MAPKs regulate filamentous growth; Hog1 MAPKs respond to high osmolarity and citric acid stress; Mpk1 MAPKs

regulate cell integrity in response to stress and low osmolarity; and Smk1 MAPKs regulate sporulation of diploid cells upon carbon or nitrogen starvation [Qi, 2005].

3.2 Snf1 kinase

Sucrose non-fermenting 1 (Snf1) kinase is a serine/threonine protein kinase conserved in plants, fungi and mammals and is essential for regulation of transcription and metabolism in response to cellular stress.

Members of the Snf1 kinase family function as heterotrimeric enzymes with three subunits named α, β and γ. The yeast genome carries *SNF1* as the only α-subunit gene, with a Ser/Thr protein kinase domain at the N-terminus and a regulatory domain at the C-terminus. The regulatory region has an autoinhibitory sequence repressing the kinase activity.

Snf1 kinase is a key regulator in the response to nutrient limitation. When cells grow in glucose, Snf1 kinase is dephosphorylated and in an inactivated form. When glucose is depleted or changed to other less efficient carbon sources such as ethanol or glycerol, Snf1 kinase is phosphorylated and activated, resulting in control of expression of transcription factors that are involved in glucose metabolism.

Under glucose starvation but not heat shock, Snf1 kinase interacts with heat shock transcription factor (HSF) and activates HSF by phosphorylation, enhancing HSF DNA binding to *SSA3* and *HSP30*, resulting in reprogramming the cellular metabolism in response to glucose availability [Hahn, 2004].

In yeast, three Snf1-activating kinases are able to phosphorylate and activate Snf1: Pak1p, Tos3p and Elm1p [Elbing, 2006]. Snf1 kinase activation is associated with ATP depletion, increase in AMP:ATP ratio and many other types of stress, such as heat shock, hypoxia and starvation. The activation of Snf1 kinase requires phosphorylation of Thr210 on its conserved activation loop and the Thr210 phosphorylation level increases significantly upon glucose depletion and nitrogen limitation.

Snf1 kinase is involved in the general stress response through regulation of protein subcellular localization. Transcriptional activator Msn2p is located mainly in the cytoplasm under normal growth conditions and its subcellular localization is regulated by the availability of carbon. Upon carbon starvation, Msn2p is activated by phosphorylation, and translocates from the cytoplasm to the nucleus. Snf1 kinase inhibits Msn2p nuclear import upon carbon starvation, but has no effect on heat or osmotic stress-induced Msn2p nuclear accumulation [Sanz, 2003]. Snf1 kinase also regulates the nuclear import of the GATA-transcription factor Gln3p, which is involved in activation of nitrogen-catabolite repressible genes expression upon carbon starvation [Bertram, 2002].

4. Research objective

The major objective of this research is to identify the molecular mechanisms and signals involved in the nuclear import and export of Hsp70 Ssa4p in budding yeast cells subjected to ethanol stress. This is accomplished by

1) identifying the sequence of events leading to the accumulation of Ssa4p in the nucleus in response to ethanol stress;

2) identifying the transport carriers required for Ssa4p nuclear import upon stress and nuclear export during recovery;

3) identifying the other components required for Ssa4p nuclear import upon ethanol stress and nuclear export during ethanol withdrawal;

4) elucidating the regulation mechanism for Ssa4p nuclear import and export upon stress;

5) examining the role of Ssa4p in cell survival and rescue.

The extended objective of this project is to study the effects of stress, nutrient state and kinase on the regulation of yeast nuclear exporters. This is accomplished by

6) investigating how various types of stresses affect the localizations of nuclear exporters;

7) detecting the kinase and the nutrient limitations that regulates the location of nuclear exporters;

8) defining the role of the kinase pathways in the regulation of exporters localization under both normal and stress conditions.

Chapters 2, 3 and 4 below will present the experimental methods and the results.

CHAPTER 2

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.

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2.1 Preface

This chapter is based on the published paper:

***Quan X**, Rassadi R, Rabie B, Matusiewicz N, Stochaj U. 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. 2004 10.1096/fj.03-0947fje* (see Appendix).

In the review of the literature (chapter 1), the classical nuclear import pathway is introduced. In this chapter we found that cNLS-mediated nuclear import is inhibited, while cytoplasmic heat shock protein 70 Ssa4p accumulates in the nuclei in response to ethanol stress. The specific goal is to define the signal and the mechanisms that promote the nuclear concentration of Ssa4p upon ethanol treatment. GFP fused Ssa4p was used to visualize the localization of the target protein. Carrier and several soluble factors required for classical nuclear import were tested for ethanol-induced Ssa4p nuclear concentration. The import complexes of Ssa4p were identified. The regulation of ethanol stress on Ssa4p nuclear import was described in detail. The importance of Pkc1p and sensors of the cell integrity pathway on Ssa4p nuclear accumulation upon ethanol stress was detected.

2.2 Abstract

Cytoplasmic proteins of the hsp70/hsc70 family redistribute in cells that have been exposed to stress. As such, the hsp70 Ssa4p of the budding yeast S. cerevisiae accumulates in nuclei when cells are treated with ethanol, whereas classical nuclear import is inhibited under these conditions. The N-terminal domain of Ssa4p, which is lacking a classical NLS, mediates nuclear accumulation upon ethanol exposure. Concentration of the Ssa4p N-terminal segment in nuclei is reversible, as the protein relocates to the cytoplasm when cells recover. Mutant analysis demonstrates that the small GTPase Gsp1p and GTPase-modulating factors are required to accumulate Ssa4p in nuclei upon ethanol stress. Moreover, we have identified the importin-beta family member Nmd5p as the nuclear carrier for Ssa4p. Ethanol treatment significantly increases the formation of import complexes containing Nmd5p and the N-terminal Ssa4p domain. Likewise, docking of the carrier Nmd5p at the nuclear pore is enhanced by ethanol. Furthermore, we show that the stressed-induced nuclear accumulation of Ssa4p depends on signaling through protein kinase C and requires sensors of the cell integrity pathway.

2.3 Introduction

Members of the hsp70 family of proteins are involved in a variety of cellular processes, including the targeting of polypeptides to different organelles, such as the nucleus [Shi, 1992; Okuno, 1993; Shulga, 1996]. In addition, hsp70s are implicated in repair of cell and tissue damage in response to heat shock and other

forms of insults [Morimoto, 1994; Benjamin, 1994; Nowak, 1994]. The yeast *Saccharomyces cerevisiae* contains six cytoplasmic hsp70s, which are members of two families. The first family consists of Ssa1p, Ssa2p, Ssa3p, and Ssa4p; proteins Ssb1p and Ssb2p are members of the second family. Gene products of the SSA family are highly homologous; however, their expression is regulated differently. Proteins Ssa1p and Ssa2p are produced under normal growth conditions; upon heat shock, SSA1 gene expression increases, and the synthesis of Ssa3p and Ssa4p is highly induced [Craig, 1994]. Like heat stress, ethanol concentrations above 4–6% lead to a strong induction of heat shock protein synthesis [Piper, 1995]. In response to stress, some cytoplasmic hsp70s accumulate in nuclei, whereas nuclear import of many other proteins and nuclear export of bulk polyA+ RNA is inhibited [Tani, 1995; Saavedra, 1999; Liu, 1996; Stochaj, 2000]. Export of mRNA encoding Ssa4p in stressed yeast cells has been studied in some detail [Saavedra, 1999; Saavedra, 1997], whereas the molecular mechanisms underlying nuclear targeting of hsp70s are poorly defined. Rat hsc70, when injected into *Xenopus oocytes*, shuttles between the nucleus and the cytosol [Mandell, 1990]. The classical bipartite NLS (cNLS) of hsc70 [Dang, 1989; Dingwall, 1991], located in the N-terminal domain, is believed to play the major role for nuclear import under normal growth conditions. However, partial deletion of the cNLS does not prevent rat hsc70 import into nuclei of *Xenopus oocytes* in unstressed cells. On the basis of these data, it was concluded that a nonclassical nuclear transport signal is present in hsc70s that promotes nuclear targeting by a pathway distinct from cNLS-mediated nuclear transport [Lamian, 1996]. Not all cytoplasmic hsp70s, however, are able to concentrate in nuclei. For instance,

Ssb1p nuclear accumulation is prevented by a nuclear export signal at the C-terminal end of the protein [Shulga, 1999].

Although previous studies with rat hsc70 have shown that the bipartite cNLS is not required for nuclear entry, these experiments did not address nuclear transport in stressed cells. Furthermore, it has not been analyzed whether a signal distinct from the cNLS mediates stress-induced nuclear transport. Likewise, it was unclear whether nuclear transport mediated by this nonclassical NLS uses components of the classical nuclear import pathway. Nuclear transport via cNLSs has been characterized in depth during the past years [Ohno, 1998; Stochaj, 1999]. The consensus model proposes that the cNLS is recognized by the dimeric cNLS-receptor importin-α/β1 and subsequently translocated across the nuclear pore complex (NPC). Srp1p, the yeast homologue of importin-α, is essential for classical nuclear import, and several soluble factors are required as well. In *S. cerevisiae*, these factors include the predominantly nuclear GTPase Gsp1p, the cytoplasmic GTPase-activating protein Rna1p, and the guanine nucleotide exchange factor Prp20p in the nucleus. GSP1, RNA1, and PRP20 are essential components, and mutations in these genes can interfere with nuclear import of cNLS-containing proteins [Corbett, 1997].

Nuclear export of bulk polyA+ mRNA and import of cNLS-containing polypeptides is sensitive to ethanol [Saavedra, 1999; Stochaj, 2000; Saavedra, 1997]. Likewise, a variety of other stress conditions interfere with classical nuclear trafficking [Stochaj, 2000]. Stress-induced inhibition of classical transport

is associated with a redistribution of soluble nuclear transport factors. In particular, the small GTPase Gsp1p, accumulated in nuclei under normal conditions, equilibrates between nucleus and cytoplasm in response to ethanol, starvation, heat stress, and hydrogen peroxide [Stochaj, 2000].

Different forms of stress can activate distinct signaling pathways in *S. cerevisiae* [Levin, 1995; Madheni, 1998; Molina, 1998]. In budding yeast, protein kinase C (Pkc1p) plays a key role in maintaining cellular integrity [Levin, 1995; Heinisch, 1999], and the single PKC1 gene is crucial for signaling through the cell integrity pathway [Perez, 2002]. Pkc1p-activated pathways are not only involved in remodeling of the cell wall during growth, they also play a role in the mating response and cell cycle progression. With respect to cell integrity, it was proposed that changes in membrane fluidity could be involved in the activation of Pkc1p [Kamada, 1995]. Furthermore, Pkc1p was also shown to regulate the relocation of nuclear proteins to the cytoplasm when cells have been exposed to high osmolarity [Nanduri, 2001]. Several cellular components can induce Pkc1p activation; this includes proteins Wsc1p (also known as Hcs77p and Slg1p) and Mid2p, sensors of cell integrity, which both reside in the cytoplasmic membrane [Heinisch, 1999; Gray, 1997; Rajavel, 1999; Ketela, 1999]. The activity of Mid2p as a sensor depends on Pmt2p, an enzyme located in the ER that is involved in Mid2p O-glycosylation [Ketela, 1999; Philip, 2001]. A deletion of PMT2 interferes with Mid2p glycosylation and signaling of the sensor to Pkc1p [Philip, 2001].

It has not been analyzed previously whether nucleocytoplasmic trafficking of heat shock proteins can be regulated by any of the components that respond to changes in cell integrity. To analyze this question, we have focused on the yeast hsp70 Ssa4p and characterized its nuclear transport route in ethanol-stressed cells. We demonstrate that Ssa4p nuclear accumulation upon stress exposure requires the nuclear carrier Nmd5p, sensors of cell integrity pathway, and Pkc1p.

2.4 Methods and materials

2.4.1 Yeast strains, media, and transformation

The following yeast strains were used: RS453 (MATa *ade2 leu2 ura3 trp1 his3*), SS328 (MATa *ade2-101 his3Δ200 lys2-801 ura3-52*), PRP20/2A (MATa *prp20-1 ade2-101 his3Δ200 lys2-801 ura3-52*), L4884 (MATa *SRP1 can1-100 ura3-1 leu2-3,112 his3-11,15 ade2-1 trp1Δ63*), L5677 (MATa *srp1-31 can1-100 ura3-1 leu2-3,112 his3-11,15 ade2-1 trp1Δ63*), SJ21R-35-4 (MATa *RNA1 ura3 leu2 ade1 rnh::URA3*), SJ21R-6-3 (MATa *rna1-1 ura3 leu2 ade1 rnh::URA3*), PSY962 (MATa *gsp1::HIS3 gsp2::HIS3 ura3-52 leu2Δ1 trp1-63* pCEN *URA3 GSP1*), Y-547 (MATα *los1::HIS3 trp1 leu2 ade2 ura3 lys1 his3*). Strains carrying mutations in one of the importin-β genes or NPC clustering strains have been described previously [Chughtai, 2001; Gao, 2003]. Yeast cells mutated in components of the cell integrity pathway were kindly provided by H. Bussey (Montreal). Cells were transformed and selected on drop-out media according to standard protocols. Expression of fusion genes was induced by overnight growth

in selective medium with 2% galactose. The growth media of yeast strains deficient in one of the components of the cell integrity pathway were supplemented with 10% sorbitol.

2.4.2 Plasmid constructions

DNA manipulations carried out in *E. coli* strain XL1-Blue were verified by sequencing. The Mut1 version of GFP was used to generate Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) [Cormack, 1996]. Fusion genes were cloned into centromeric plasmids, which carried the *URA3* or *LEU2* marker and the inducible *GAL1* promoter. A bacterial expression plasmid encoding His_6-tagged Nmd5p was provided by G. Schlenstedt (Homburg). The His_6-Nmd5p coding sequence was cloned into a yeast expression vector carrying the *GALS* promoter [Mumberg, 1994].

2.4.3 Plasmid shuffling

The mutant allele *gsp1-2*, encoded by a centromeric plasmid carrying the *TRP1* marker, was introduced into strain PSY962 [Wong, 1997] following standard procedures. For comparison, wild-type *GSP1* was expressed from the same plasmid vector.

2.4.4 Western blot analysis and quantitation of ECL signals

Proteins were detected by Western blotting with antibodies against GFP (diluted 1:200, Clontech, Palo Alto, CA) or the His_6-epitope (1:100, Santa Cruz Biotechnology, CA) as described [Chu, 2001]. Signals obtained after ECL

(Pierce, Rockford, IL) were quantified by densitometry of the films using the software "Spot Density Tools" as suggested by Alpha Innotech Corporation (San Leandro, CA). In brief, density measurements were carried out for each area of interest and corrected for background. All of the samples quantified for an individual experiment were present on the same filter. Filters were exposed to film for different times to ensure that the strength of the brightest signal was below the saturation level of the film.

2.4.5 Fluorescence microscopy of yeast cells

Yeast cells were grown and exposed to stress as detailed in the text. Cells were fixed with 3.7% formaldehyde for 10 min at room temperature, washed in 0.1 M potassium phosphate pH 6.5 and immobilized on multiwell slides coated with polylysine. Slides were immersed in ice-cold methanol (6 min) followed by ice-cold acetone (30 s). Slides were dried, washed once in PBS, and wells were incubated with 1μg/ml DAPI (4',6-diamidino-2-phenylindole) for 1 min. Slides mounted in Vectashield (Vector Laboratories, Burlingame, CA) were sealed with rubber cement or nail polish. Samples were analyzed with a Nikon Optiphot at 1,000 X magnification. Cells highly overexpressing GFP or one of its fusion proteins were excluded from further analysis to avoid the nonlinear range of fluorescence signals. For each set of experiments, images wereexposed for the same time, and negatives were processed with Photoshop 5.5.

2.4.6 Nuclear import of cNLS-containing proteins

Classical nuclear transport was analyzed essentially as described [Shulga, 1996]. The reporter protein NLSGFP carries the simple SV40-NLS; inhibition of classical nuclear import increases levels of NLS-GFP in the cytoplasm [Stochaj, 2000].

2.4.7 Isolation of protein complexes containing His_6-Nmd5p and Ssa4p(1-236)-GFP

Yeast cells were grown at room temperature in drop-out medium containing 2% galactose to an OD_{600} of 1.0 to 1.3. Treatment with 10% ethanol was for 10 min at room temperature. Cells were collected by centrifugation, washed once in distilled water and stored at −70°C. Yeast cells equivalent to 10 OD_{600} units were lysed with glass beads in buffer A (20 mM Pipes/KOH, 250 mM sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate, pH 6.8, 1% Triton X-100, 0.5 mM DTT, 1 mM PMSF, 5 mM benzamidine, and protease inhibitors [antipain, aprotinin, chymostatin, leupeptin and pepstatin; each at 1 µg/ml]). Supernatants obtained after 2 min centrifugation (microfuge, 15,000 rpm, 4°C) were incubated with Ni-NTA agarose (Qiagen, Hilden, Germany) with gentle agitation for 2 h at 4°C. The resin was collected by centrifugation and washed three times with 5 mM imidazole in buffer A without Triton X-100 and DTT. For Western blot analysis bound material was eluted by boiling for 10 min in gel sample buffer.

2.4.8 Purification of GST-Gsp1pQ71L and isolation of His_6-Nmd5p/ Ssa4p(1-236)-GFP protein complexes

GST-Gsp1pQ71L (plasmid kindly provided by G. Schlenstedt) was purified from *E. coli* using standard procedures. For affinity purification of Nmd5p/Ssa4p complexes 5 μM GSTGsp1pQ71L was added together with 1 mM GTP before the purification of protein complexes by metal affinity chromatography.

2.5 Results

2.5.1 The cytoplasmic hsp70 Ssa4p translocates rapidly into nuclei upon exposure to ethanol

In the yeast *S. cerevisiae* as in other eukaryotes, cytoplasmic hsp70s accumulate in nuclei in response to various stresses. To follow the distribution of the hsp70 Ssa4p, we have fused *Aequorea victoria* green fluorescent protein (GFP) to different portions of the heat shock protein. In wild-type cells, 10% ethanol triggered the nuclear accumulation of GFP-Ssa4p(16-642), which was concentrated in nuclei when analyzed after a 10-min treatment (Fig 2.1). Thus, our experiments established that Ssa4p, a member of the cytoplasmic *SSA*-protein family, accumulates in nuclei after a short incubation with ethanol.

2.5.2 The N-terminal domain of Ssa4p promotes nuclear accumulation in stressed cells

Having shown that Ssa4p concentrates in nuclei of ethanol-treated cells, we next generated a fusion between the N-terminal 236 amino acid residues of Ssa4p and GFP. This fusion protein, termed Ssa4p(1-236)-GFP, does not contain the bipartite cNLS present in Ssa4p. With a molecular mass greater than 50 kDa,

Ssa4p(1-236)-GFP is too large to efficiently diffuse across the nuclear envelope [Chughtai, 2001]. Under normal growth conditions, Ssa4p(1-236)-GFP was cytoplasmic and nuclear in wild-type cells (Fig 2.1B). The same distribution was obtained under nonstress conditions with several yeast strains, including various mutants (Fig 2.5, 2.6 and not shown). Like GFP-Ssa4p(16-642), Ssa4p(1-236)-GFP accumulated in nuclei upon 10 min exposure to ethanol (Fig 2.1B). For comparison of Ssa4p(1-236)-GFP trafficking with classical import, the nuclear reporter protein NLS-GFP, which carries the simple SV40-NLS fused to GFP, was also located. Classical nuclear import was inhibited efficiently by treating cells with ethanol. As a consequence, increased levels of NLS-GFP, which is normally concentrated in nuclei (Fig 2.1C), were detected in the cytoplasm (Fig 2.1C). This inhibition of classical nuclear import in response to ethanol was also seen for mutant strains used in our studies, demonstrating that the various mutations in nuclear transport factors do not obstruct the NPC in response to stress exposure (Fig 2.2 and data not shown). In contrast to GFPSsa4p(16-642), Ssa4p(1-236)-GFP, and NLS-GFP, the GFP-tag was not affected by stress; this protein was nuclear as well as cytoplasmic under all conditions (not shown).

Other forms of insults, such as osmotic or oxidative stress, have been shown to relocate proteins to the nucleus. For instance, the HOG pathway can be activated by sodium chloride, resulting in rapid Hog1p nuclear accumulation upon mild, but not severe, osmotic stress [Ferrigno, 1998; Reiser, 1999; Wuytswinkel, 2000]. However, neither mild nor severe osmotic stress was effective in concentrating Ssa4p(1-236)-GFP in nuclei (Fig 2.1B, Table 2.1). In addition, several different

conditions were tested for hydrogen peroxide, an agent that induces the nuclear localization of the transcription factor yAP-1 and inhibits classical nuclear import [Liu, 1996; Kuge, 1997]. Like osmotic stress, oxidative stress failed to accumulate Ssa4p(1-236)- GFP or GFP-Ssa4p(16-642) in nuclei (Fig 2.1B, Table 2.1 and data not shown).

2.5.3 The Star-sequence does not promote nuclear accumulation in ethanol-treated cells

We have previously identified a short hydrophobic segment of Ssa4p, comprising amino acid residues 162 to 171, as a signal that is sufficient to locate the cytoplasmic protein β-galactosidase to nuclei of starving cells [Chughtai, 2001]. This peptide, termed Star (for starvation), was tested for its capacity to accumulate a Star-β-galactosidase fusion protein in nuclei. However, ethanol treatment did not stimulate nuclear import of Star-β-galactosidase in wild-type cells (Fig 2.3).

2.5.4 Nuclear accumulation of Ssa4p(1-236)-GFP in response to ethanol is reversible

To determine whether ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP is reversible, cells were treated with 10% ethanol for 10 min and transferred for stress recovery to ethanol-free medium containing glucose, which represses de novo transcription of the *SSA4(1-236)-GFP* gene. Upon 30 min of incubation, Ssa4p(1-236)-GFP nuclear accumulation was reduced, and 2 h after transfer to the fresh medium the protein had equilibrated between nucleus and cytoplasm (Fig

2.4A, B). To exclude the possibility that Ssa4p(1-236)-GFP in the cytoplasm represents protein translated from pre-existing mRNA, the experiment was repeated in the presence of cycloheximide. The protein synthesis inhibitor was present throughout the stress and recovery period (Fig 2.4C). Although we detected a larger percentage of cells with weak nuclear accumulation (N≥C) after 1 h recovery, results obtained for 2 h recovery were similar to those of samples incubated without the inhibitor. Thus, the concentration of Ssa4p(1-236)-GFP in nuclei of stressed cells is a reversible process, suggesting that the protein shuttles between nucleus and cytoplasm.

2.5.5 Nuclear accumulation of Ssa4p is independent of Srp1p but requires Gsp1p, Rna1p, and Prp20p

Several soluble factors are required for classical nuclear transport, and mutations in *SRP1*, *GSP1*, *RNA1* and *PRP20* are known to prevent cNLS-dependent nuclear import under nonpermissive conditions [Corbett, 1995; Schlenstedt, 1995; Koepp, 1996; Corbett, 1997]. To test the role of these components in nuclear transport of the N-terminal domain of Ssa4p, we incubated conditionally lethal mutants at the nonpermissive temperature to inactivate the mutant transport factors. These pretreated cells failed to import cNLS-containing substrates [Corbett, 1995; Schlenstedt, 1995; Koepp, 1996; and data not shown].

Following incubation at the nonpermissive temperature, cells were treated with ethanol (Fig 2.5). As described above, 10 min exposure to ethanol accumulated Ssa4p(1-236)-GFP in nuclei of wild-type cells. This was observed for all of the

parental wild-type strains used in this study (Fig 2.5A–D). Likewise, after preincubation at the nonpermissive temperature, cells carrying the *srp1-31* allele concentrated Ssa4p(1-236)-GFP in nuclei in response to ethanol (Fig 2.5A). Thus, Srp1p, the α-subunit of the cNLS-receptor, is not essential for stress-mediated nuclear import of Ssa4p(1-236)-GFP in response to ethanol. These data are consistent with the idea that nuclear import of the N-terminal domain of Ssa4p is not mediated by the classical import route, but by a specialized pathway.

The same strategy as described for Srp1p was employed to determine whether Gsp1p, Rna1p, or Prp20p are involved in nuclear import of Ssa4p(1-236)-GFP (Fig 2.5B–D). After pretreatment of cells at 37°C and subsequent exposure to ethanol, parental wild-type cells accumulated Ssa4p(1- 236)-GFP in nuclei (Fig 2.5B–D). In contrast, Ssa4(1-236)-GFP failed to concentrate in nuclei of *gsp1-2*, *rna1-1* or *prp20-1* cells (Fig 2.5B–D), demonstrating that Gsp1p, Rna1p, and Prp20p are required for stress-dependent nuclear import of the N-terminal domain of Ssa4p.

2.5.6 Nuclear accumulation of Ssa4p upon ethanol exposure requires the carrier Nmd5p

Members of the importin-β family are involved in nucleocytoplasmic trafficking of a variety of cargos (reviewed in 46). Moreover, nuclear transport mediated by these carriers depends on the small GTPase Gsp1p and its interacting factors. To test the potential role of importin-β proteins in Ssa4p(1-236)-GFP nuclear transport wild-type and mutant cells carrying a knockout in one of the importin-β genes were incubated with ethanol for different periods of time (Fig 2.6A–C).

This strategy identified Nmd5p as the carrier necessary for the rapid nuclear accumulation of Ssa4p(1- 236)-GFP in response to ethanol treatment. In contrast to the inactivation of *NMD5*, mutations in several other importin-β family members did not prevent Ssa4p(1-236)-GFP from concentrating in the nucleus (Fig 2.6A, C). Like the N-terminal portion of Ssa4p, GFP-Ssa4p(16-642) also required the carrier Nmd5p to accumulate in nuclei of cells that had been exposed to ethanol (Fig 2.6B). In further experiments, we determined the kinetics of Ssa4p(1-236)-GFP nuclear concentration. All of the mutant strains, except *nmd5::TRP1*, accumulated Ssa4p(1-236)-GFP in nuclei after 2 min incubation with 10% ethanol (Fig 2.6C). In the presence of ethanol, this nuclear accumulation persisted for at least 60 min.

Although deficient in nuclear import of Ssa4p upon ethanol treatment, *nmd5::TRP1* cells were not defective in classical nuclear import (Fig 2.2). NLS-GFP accumulated in nuclei of control cells and relocated to the cytoplasm upon exposure to ethanol. As expected, in control experiments, *nmd5::TRP1* cells were unable to accumulate Hog1p-GFP in nuclei in response to osmotic stress (Fig 2.7).

2.5.7 Effect of ethanol stress on the viability of yeast cells

We further characterized the toxicity of ethanol by monitoring the viability of wild-type and *nmd5::TRP1* strains that had been treated with alcohol. After exposure to 10% ethanol for 10 min, cells were immediately diluted and plated out. The number of colonies formed was compared with untreated control cells.

For wild-type and mutant cells synthesizing Ssa4p(1- 236)-GFP, the number of colonies was reduced to 60–70% of the unstressed controls (Fig 2.8). Similar results were obtained for untransformed cells, in line with the idea that the majority of cells recover from ethanol-induced damage (Fig 2.8, and data not shown).

2.5.8 Nmd5p and Ssa4p interact in growing yeast cells

Because *nmd5::TRP1* cells failed to accumulate Ssa4p(1-236)-GFP in nuclei in response to ethanol treatment, it was conceivable that Nmd5p and Ssa4p can associate to form import complexes. To analyze such a potential interaction between Nmd5p and Ssa4p(1-236)-GFP, His_6-tagged Nmd5p and Ssa4p(1-236)-GFP were simultaneously synthesized in yeast. Metal affinity chromatography was used to purify protein complexes that contained His_6-Nmd5p and Ssa4p(1-236)-GFP (Fig 2.9A, part a). Under these conditions, similar amounts of His_6-Nmd5p were purified from control and stressed cells (Fig 2.9A, part c and f). The interaction between His_6-Nmd5p and Ssa4p(1-236)-GFP was not only observed when both proteins were synthesized in the same yeast cell, both proteins also copurified when yeast extracts containing either protein were mixed before affinity purification (Fig 2.9A, part b). Control experiments demonstrated the specificity of this interaction, as Ssa4p(1-236)-GFP did not bind to the resin in the absence of His_6-Nmd5p (Fig 2.9A, part a).

Nuclear import complexes containing a member of the importin-β family of carriers can be dissociated by the GTP-bound form of Ran/Gsp1p [Ohno, 1998].

When purified GST-Gsp1p- Q71L, a mutant that mimics the GTP-bound form of Gsp1p, was added to the binding assay, the purification of His_6-Nmd5p was not affected (Fig 2.9A, part e). When the amounts of His_6-Nmd5p purified under different conditions were compared, a maximum variation of 3 ± 3.7% was observed (Fig 2.9A, part f). By contrast, the levels of Ssa4p(1-236)-GFP that copurified with His_6- Nmd5p were drastically reduced by GST-Gsp1pQ71L (Fig 2.9A, part d, f; ** $P<0.01$). This effect of GST-Gsp1pQ71L on the formation of His_6-Nmd5p/Ssa4p(1-236)-GFP complexes was detected for unstressed as well as ethanol-treated cells. As shown in Fig 2.9, exposure to ethanol was not a prerequisite for the association between His_6-Nmd5p and Ssa4p(1-236)-GFP. However, the amount of Ssa4p(1-236)-GFP complexed with His_6-Nmd5p was increased significantly by incubation with ethanol (Fig 2.9A, part f; x, $P=0.04$).

In most of our experiments anti-GFP antibodies recognized two major bands in cells synthesizing Ssa4p(1-236)-GFP, but the faster migrating protein did not associate with His_6- Nmd5p. This faster migrating band could be a proteolytic product of Ssa4p(1-236)-GFP. Alternatively, the higher molecular mass form might have been modified post-translationally. At present, we cannot distinguish between these possibilities.

The specificity of the Nmd5p/Ssa4p(1-236)-GFP interaction was further analyzed in additional control experiments, showing that the GFP-tag did not associate with His_6-Nmd5p (Fig 2.9B). When cells were cotransformed with plasmids encoding His_6-Nmd5p and GFP, only low-level synthesis of GFP was obtained (not shown).

Therefore, crude cell extracts containing either His_6- Nmd5p or GFP were mixed before affinity chromatography. GFP did not interact with Nmd5p (Fig 2.9B, part b), whereas Ssa4p(1-236)-GFP bound to the carrier under these conditions (Fig 2.9A, part b).

2.5.9 Ethanol stimulates the association of Nmd5p-GFP with NPCs

Results described in the previous section demonstrate that the association between Nmd5p and Ssa4p(1-236)-GFP was stimulated by the incubation with ethanol. To test whether a subsequent step of nuclear import, i.e., binding of Nmd5p to NPCs, is regulated by ethanol, we have used two different NPC clustering strains, *rat2-1* and *nup133*Δ. Both strains form NPC clusters when incubated at room temperature, but mRNA export and classical nuclear import are not affected [Levin, 1995 and references therein]. We located NPC clusters in these cells by staining with mab414, an antibody that binds to FXF-containing nucleoporins (Fig 2.10A, B). Binding of Nmd5p-GFP to clusters was then quantified for different experimental conditions. In untreated controls, about one-third of the cells displayed association of Nmd5p-GFP with NPC clusters (Fig 2.10B). In response to ethanol, however, the interaction of Nmd5p-GFP with NPC clusters was significantly increased in both strains. This enhanced binding of Nmd5p-GFP was observed after 2 min exposure to ethanol, and similar results were obtained after 10 min incubation. These results are comparable with the kinetics of Ssa4p(1-236)-GFP nuclear accumulation in various strains carrying the *NMD5* wild-type allele, which concentrated most of Ssa4p(1-236)-GFP in nuclei after 2 min exposure to ethanol (Fig 2.6C). In control experiments, binding of Nmd5p-GFP to

NPCs was tested upon osmotic stress. As expected [Heinisch, 1999], 10 min incubation with 0.4 M NaCl significantly increased the association of Nmd5p with NPC clusters (Fig 2.10B).

2.5.10 Stress-induced nuclear accumulation of Ssa4p(1-236)-GFP relies on Pkc1p

Given the effect of ethanol on membrane fluidity and the important role of Pkc1p, Mid2p, and Wsc1p in the cell integrity pathway and of Pmt2p in regulating Mid2p sensor activity, it was possible that these components are involved in the targeting of Ssa4p to the nucleus of ethanol treated cells. We have tested this hypothesis by analyzing the nuclear accumulation of Ssa4p(1- 236) in knockout mutants *pkc1*Δ, *wsc1*Δ, *mid2*Δ, *pmt2*Δ, and the double mutant *wsc1*Δ *mid2*Δ. In all of these mutants, the nuclear accumulation of Ssa1p(1-236)-GFP after ethanol treatment was severely impaired (Fig 2.11A, B). In particular, mutants *pkc1*Δ, *mid2*Δ, and the double mutant *wsc1*Δ *mid2*Δ failed to respond properly to ethanol stress, whereas a minor nuclear accumulation of Ssa4p(1-236)-GFP was observed for *wsc1*Δ (Fig 2.11B). Unlike these mutants, parental wildtype strains did not show a defect in the ethanol-induced nuclear accumulation of the reporter protein (Fig 2.11 and data not shown).

2.6 Discussion

Ssa4p, a member of the cytoplasmic hsp70 family, concentrates in the nucleus when cells are exposed to ethanol. By contrast, other types of insults, such as

osmotic or oxidative stress, fail to induce the nuclear accumulation of Ssa4p. These results suggest that ethanol produces a specific cellular response for which Ssa4p is a downstream target.

With the experiments described here, we have demonstrated that the N-terminal 236 amino acid residues of Ssa4p are sufficient to mediate the nuclear accumulation of the non-nuclear passenger GFP when cells are exposed to ethanol. This N-terminal portion of Ssa4p does not contain a cNLS, consistent with the idea that a nonclassical signal mediates stress-induced nuclear import. In line with this hypothesis, ethanol-induced nuclear import of Ssa4p does not depend on Srp1p, the α-subunit of the cNLS-receptor, but requires the importin-β Nmd5p. Although Ssa4p nuclear import caused by ethanol has unique requirements, it shares components with the classical import machinery. In particular, the Gsp1p-GTPase system plays a role in nuclear import, as well as nuclear export of a variety of substrates. Our analyses of conditionally lethal mutants reveal that Gsp1p and its modulating factors Rna1p and Prp20p are necessary to accumulate Ssa4p(1-236)-GFP in nuclei when cells have been treated with ethanol.

A hallmark of stress-dependent inhibition of classical nuclear import is the collapse of the Gsp1p concentration gradient. Under normal growth conditions, Gsp1p is predominantly nuclear, whereas ethanol stress dissipates the Gsp1p concentration gradient across the nuclear envelope [Stochaj, 2000]. However, cells exposed to ethanol still carry out the Gsp1p GTPase cycle [Saavedra, 1999].

Differences in the requirement for the mammalian Gsp1p homologue Ran with respect to various nuclear transport pathways have been reported [Michael, 1997; Kose, 1997; Kose, 1999; Ribbeck, 1999; Yokoya, 1999; reviewed in Stochaj, 1999]. As such, importin-β can be translocated through NPCs in a Ran-independent fashion [Kose, 1997; Kose, 1999]. On the basis of these observations, we propose that the stress-induced nuclear accumulation of cytoplasmic hsp70s requires Gsp1p, but not a steep concentration gradient of the GTPase. It is likely that the amount of Gsp1p-GTP left in the nucleus is sufficient to support Ssa4p(1-236)-GFP nuclear import in ethanol-treated cells.

The complete GTPase cycle, with both Gsp1p in its GTP-bound and GDP-bound form, is necessary for Ssa4p(1-236)-GFP nuclear transport. These results are in agreement with the requirement for the carrier Nmd5p for Ssa4p import into nuclei of ethanol-treated cells. Like other importin-β family members, Nmd5p depends on the Gsp1p GTPase cycle to translocate cargo across the nuclear pore complex. We have now identified the hsp70 Ssa4p as a novel transport substrate for Nmd5p whose nuclear import is induced by ethanol exposure. Furthermore, our experiments show that the N-terminal part of Ssa4p is sufficient to promote the specific association with Nmd5p in growing cells. This association can be disrupted by Gsp1p- GTP, demonstrating that the interaction Nmd5p/Ssa4p(1-236)-GFP has the properties of a bona fide import complex.

While Nmd5p and Ssa4p(1-236)-GFP can associate in unstressed cells, ethanol treatment stimulates significantly the binding of Ssa4p(1-236)-GFP to Nmd5p.

This increase in import complexes is likely to contribute to the nuclear accumulation of Ssa4p(1-236)-GFP. In addition to the stimulation of import complex formation, subsequent steps in Ssa4p(1-236)-GFP nuclear transport may also be affected by ethanol. Indeed, we found the interaction of Nmd5p with NPCs to be up-regulated significantly by exposure to ethanol. Taken together, our results suggest that the increase in import complex generation as well as the enhanced binding of Nmd5p to NPCs stimulate the nuclear accumulation of Ssa4p(1-236)-GFP.

As various alcohols affect the fluidity of membranes and potentially also the diffusion limit of the NPC [Shulga, 2003], it might be assumed that leakage across the nuclear envelope plays a role in the relocation of proteins between nucleus and cytoplasm. As such, Ssa4p(1-236)-GFP may traverse the NPC by diffusion and accumulate in nuclei because of retention. However, several lines of evidence argue against such a simple diffusion/retention mechanism. For instance, the use of ethanol in wild-type cells failed to increase the diffusion limit of NPCs [Shulga, 2003]. More importantly, diffusion/retention is unlikely to depend on the Gsp1p-GTPase system and the carrier Nmd5p, components which we have shown to be required for the ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP. Although we do not rule out the possibility that retention contributes to the nuclear concentration of Ssa4p(1-236)-GFP after its import, our results clearly demonstrate that nuclear accumulation requires a nonclassical nuclear transport pathway.

One potential complication of the experiments discussed here is that the expression levels of genes encoding GFP-fusions might influence the localization of reporter proteins. Such a process would affect, in particular, proteins of low abundance, and it might result in their leakage across the nuclear envelope. However, in the case of cytoplasmic hsp70s, even normal growth conditions require that at least one member of the Ssa protein family is present at high concentrations to ensure cell viability [Craig, 1994 and references therein]. Furthermore, as discussed above, Ssa4p(1-236)-GFP relocation via a leakage/nuclear retention mechanism is unlikely, as the Ssa4p(1-236)-GFP nuclear accumulation depends on a nonclassical transport route. In the case of NLS-GFP, the reporter protein for classical nuclear import, previous studies have shown that under the conditions used in our experiments NLS-GFP is a reliable tool to monitor nuclear transport in growing yeast cells [Shulga, 1996; Stochaj, 2000; Shulga, 1999; Chughtai, 2001 and references therein].

Although cells that do not synthesize Nmd5p fail to import Ssa4p into nuclei of ethanol-treated cells, inactivation of the *NMD5* gene does not reduce the viability of cells upon ethanol exposure. One possible explanation for this result could be that ethanol-induced damage triggers a variety of repair processes, one of which is Ssa4p targeting to nuclei. However, it is feasible that other responses are also necessary to recover from stress and restore the normal cell physiology. As such, it is conceivable that ethanol will affect the fluidity of membranes and the proper function of membrane-associated proteins. If this is the case, nuclear transport of

Ssa4p and the presence of Nmd5p may not be the limiting factors that regulate the survival of ethanolstressed cells.

As discussed above, our experiments clearly show that the cNLS of Ssa4p is not required for ethanol-induced nuclear accumulation. We have further attempted to define the minimal region within the N-terminal domain of Ssa4p that is sufficient for nuclear localization in stressed cells. However, smaller segments of Ssa4p were not stable when synthesized in yeast, which prevented the analysis of their potential targeting function (not shown). Nevertheless, we have tested whether the Star-sequence of Ssa4p, which can direct β-galactosidase to the nucleus upon starvation [Chughtai, 2001], mediates nuclear accumulation in ethanol-treated cells. Unlike Ssa4p(1-236)- GFP, Star-β-galactosidase failed to concentrate in nuclei of cells treated with ethanol. These results suggest that the N-terminal domain of Ssa4p provides more than one nuclear import signal for nuclear accumulation in response to distinct types of stress. The presence of multiple import signals with different transport characteristics has been reported previously for ribosomal proteins [Rudt, 2001]. The use of divergent transport sequences after exposure to different environmental challenges may offer the advantage to control the stress response and fine-tune the nucleocytoplasmic distribution of Ssa4p, according to the physiological requirements of the cell.

Our data demonstrate that ethanol, but not other environmental stresses like oxidants or osmotic stress, promotes nuclear import of Ssa4p. This indicates that a specific response is triggered in cells that have been treated with ethanol, distinct

from the signaling events activated upon treatment with hydrogen peroxide or salt. Indeed, we have shown here that Ssa4p(1-236)-GFP fails to accumulate in nuclei of mutants that are lacking Pkc1p or one of the major sensors of cell integrity, that is, Wsc1p and Mid2p. Both Wsc1p and Mid2p are proteins of the cytoplasmic membrane where they act as upstream regulatory sensors that can activate Pkc1p [reviewed in Heinisch, 1999]. Wsc1p and Mid2p have been proposed to have partially overlapping functions, with Wsc1p playing a major role in vegetative growth, whereas Mid2p is mostly involved in mating [Heinisch, 1999; Rajavel, 1999; Ketela, 1999]. Our results show that Wsc1p and Mid2p are both required for Ssa4p(1-236)-GFP nuclear accumulation in ethanol-stressed cells during vegetative growth. Mid2p seems to play a more important role in this process, as *mid2*Δ mutants were more severely affected in nuclear import of the reporter protein. Like *mid2*Δ, the mutant *pkc1*Δ failed to respond properly to ethanol stress and did not concentrate Ssa4p(1-236)-GFP in nuclei. On the basis of these results, we propose that Mid2p and Wsc1p sense ethanol-induced changes in the plasma membrane, such as alterations of the bilayer fluidity. This signal is then transmitted to Pkc1p, and Ssa4p could be a downstream target of this pathway, which accumulates in nuclei upon activation of the protein kinase. Future experiments will have to define the molecular components that link Pkc1p activation to the concentration of Ssa4p in nuclei.

2.7 Acknowledgements

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Table 2.1 Localization of Ssa4p(1-236)-GFP upon exposure to different stresses.

Conditions	[%] N>C	N≥C	N+C
Control	11.0 +/- 3.0	0	89.0 +/- 3.0
10 % EtOH, 10 min	86.7 +/- 3.2	0	13.3 +/- 3.2
0.3 mM H_2O_2, 10 min	14.0 +/- 15.1	0	86.0 +/- 15.1
0.3 mM H_2O_2, 60 min	1.7 +/- 1.5	1.0 +/-1.7	97.3 +/- 0.6
0.3 mM H_2O_2, 120 min	2.0 +/- 2.4	1.0 +/- 1.7	97.0 +/- 3.0
2 mM H_2O_2, 10 min	12.7 +/- 12.1	0	87.3 +/- 12.1
0.4 M NaCl, 1 min	0	3.7 +/- 2.5	96.3 +/- 2.5
0.4 M NaCl, 10 min	7.6 +/- 6.7	2.7 +/- 4.6	89.7 +/- 2.1
1.4 M NaCl, 45 min	5.0 +/- 3.0	0	95.0 +/- 3.0
1.4 M NaCl, 60 min	1.7 +/- 1.5	0	98.3 +/- 1.5
1.4 M NaCl, 120 min	0.3 +/- 0.6	0	99.7 +/- 0.6

The reporter protein was located by fluorescence microscopy in wild type cells. At least 100 cells expressing the reporter gene were monitored in each experiment. Numbers represents means and S.D. of three independent experiments.

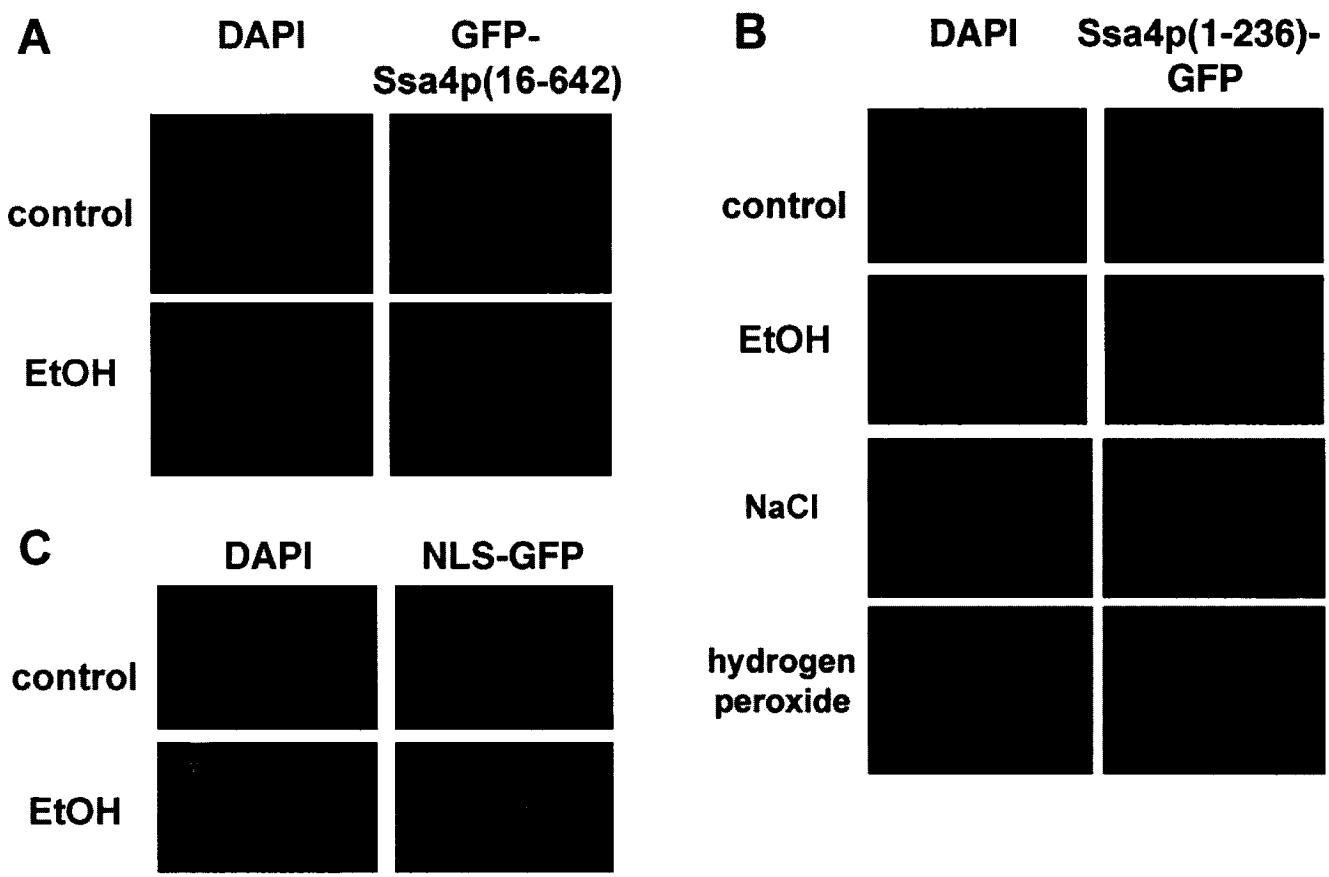

Figure 2.1 Stress induced relocation of the cytoplasmic hsp70 Ssa4p and NLS-GFP.

Wild type yeast cells synthesizing Ssa4p fusion proteins or NLS-GFP were grown overnight in drop out medium containing 2% galactose. In fixed cells DNA was visualized with DAPI and fusion proteins were localized by fluorescence microscopy. The experiment was carried out with (A) GFP-Ssa4p(16-642), (B) Ssa4p(1-236)-GFP and (C) NLS-GFP. GFP-containing reporter proteins were located in control cells or after 10 min exposure to 10 % ethanol, 0.4 M NaCl or 0.3 mM hydrogen peroxide.

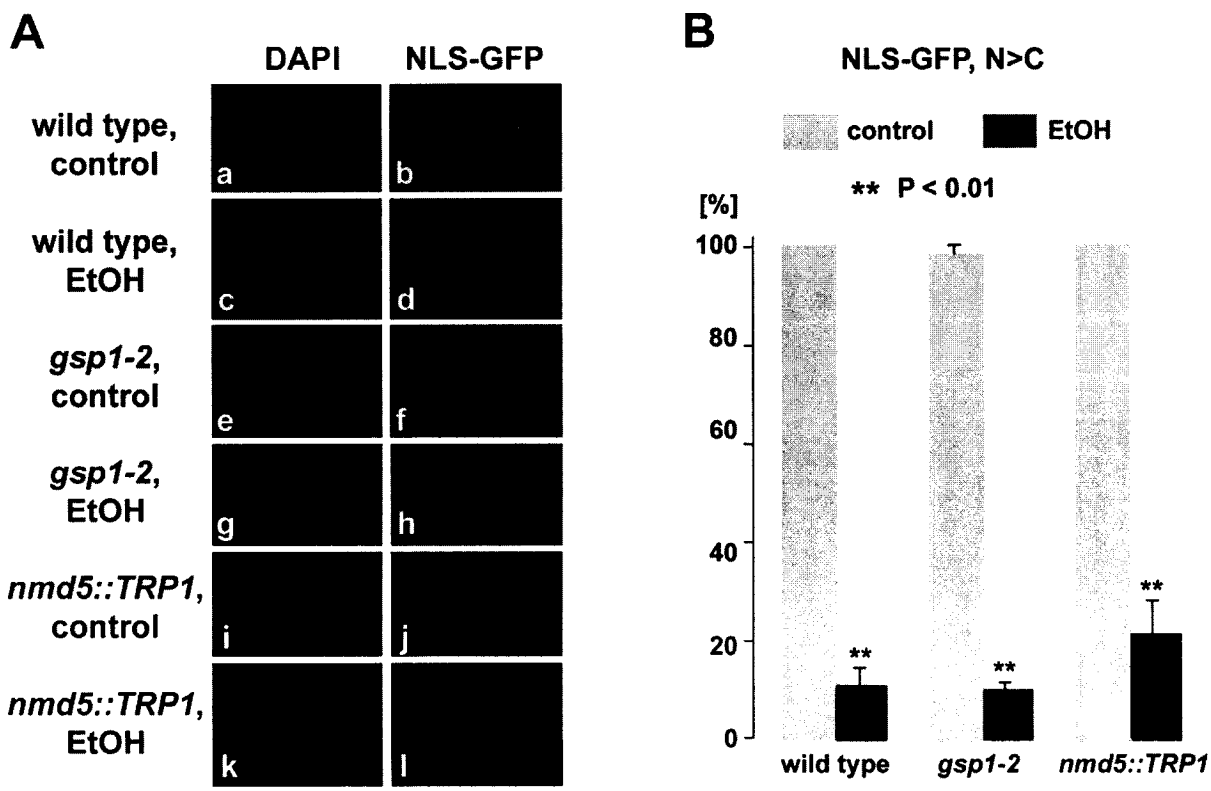

Figure 2.2 Inhibition of classical nuclear import in wild type and mutant yeast strains treated with ethanol.

(A) NLS-GFP was localized by fluorescence microscopy in wild type cells (a-d), gsp1-2 (e-h) and nmd5::TRP1 (i-l) for nonstress conditions (b, f, j) and upon 10 min incubation with 10% ethanol (d, h, l). After fixation of the cells DNA was stained with DAPI and NLS-GFP was located by fluorescence microscopy. (B) At least 65 cells were evaluated in three independent experiments for control and stress conditions (EtOH). Means and S.D. are shown.

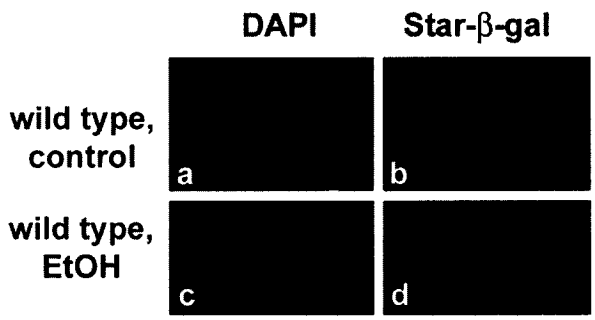

Figure 2.3 The Star-sequence does not promote nuclear accumulation in ethanol treated cells.

Wild type cells synthesizing Star-β-galactosidase (Star-β-gal) were tested for the localization of the reporter protein before (b) and after (d) 10 min incubation with 10% ethanol. Nuclei were visualized with DAPI and Star-β-galactosidase was detected by indirect immunofluorescence with antibodies against β-galactosidase.

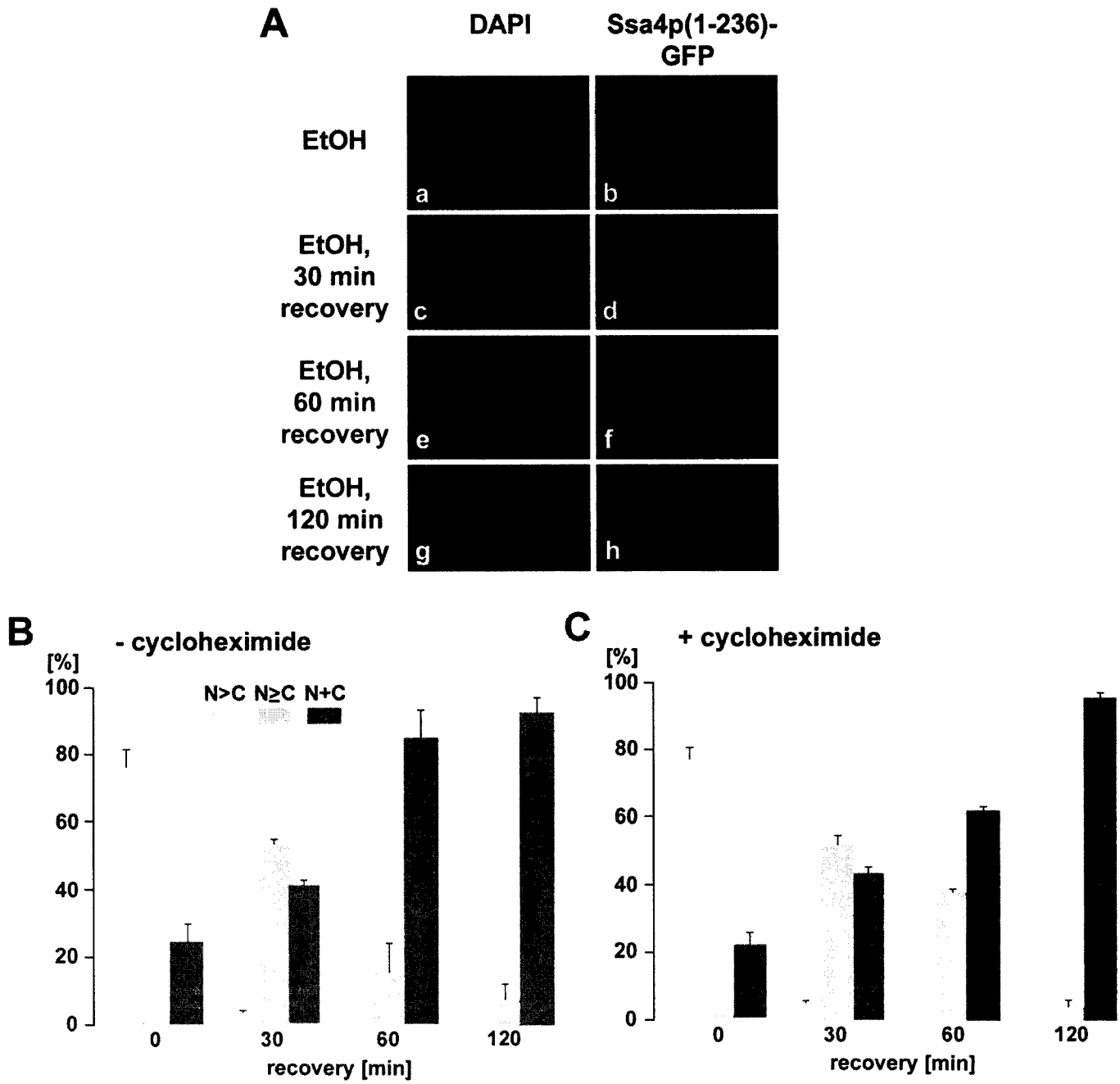

Figure 2.4 Ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP is reversible.

Wild type cells synthesizing Ssa4p(1-236)-GFP were exposed to 10 % ethanol for 10 min at room temperature. Cells were immediately transferred to ethanol free medium containing glucose and incubated at room temperature for the times indicated. (A) Fluorescence microscopy of stressed and recovering cells was carried out as described for Fig 1.1 (B) The localization of Ssa4p(1-236)-GFP was determined for different times of recovery. At least 100 cells synthesizing Ssa4p(1-236)-GFP were analyzed in each of three independent experiments. Means and S.D. are shown for the different time points analyzed. (C) The experiment shown in part B was carried out in the presence of cycloheximide. The protein synthesis inhibitor was present at a final concentration of 100 µg/ml during the incubation with ethanol and throughout the recovery period. N>C, nuclear accumulation; N≥C, weak nuclear accumulation; N+C, equal fluorescence signals were discovered in nucleus and cytoplasm.

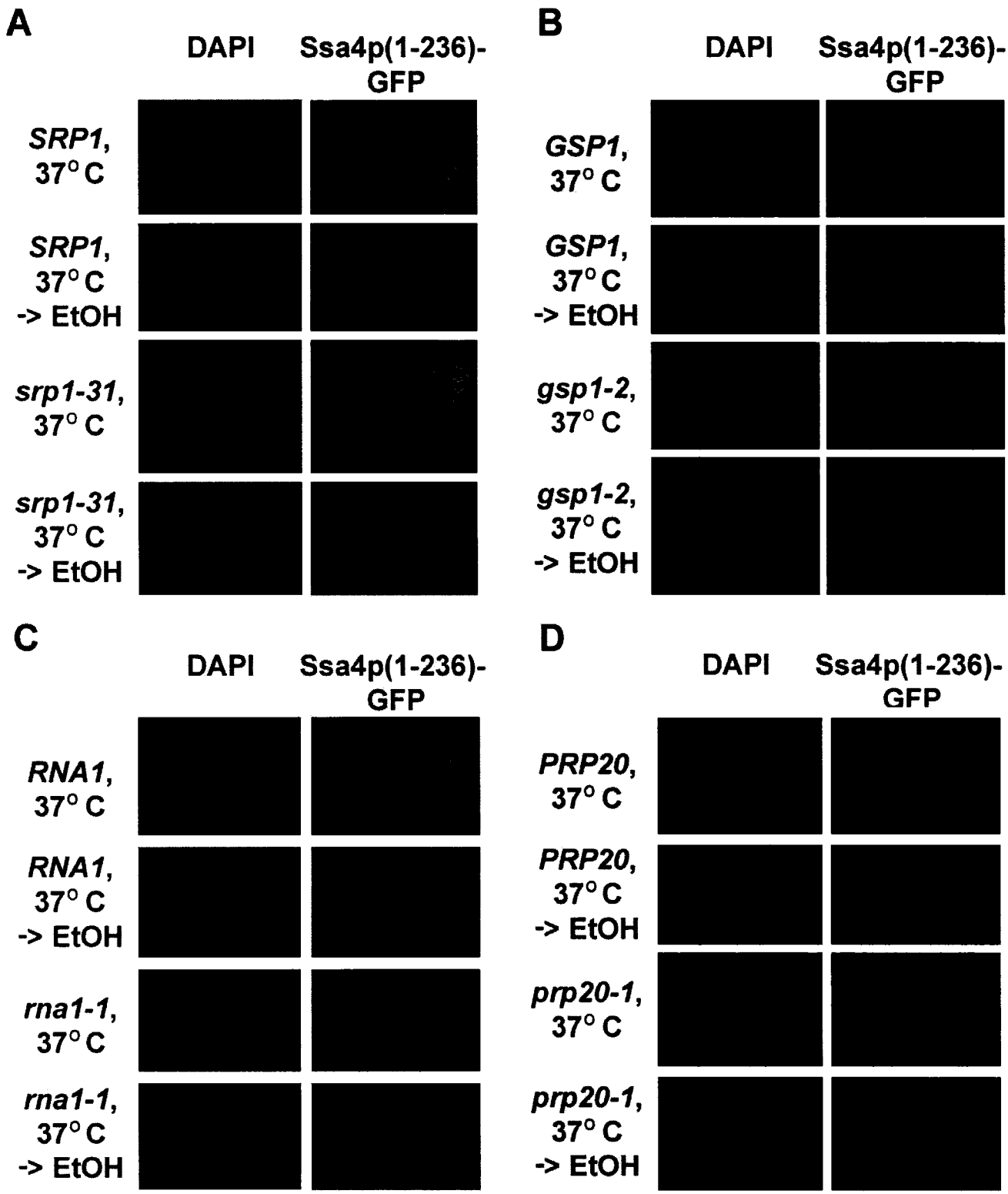

Figure 2.5 Localization of Ssa4p-GFP in yeast strains carrying conditionally lethal alleles of *SRP1, GSP1, RNA1, PRP20.*

Parental wild type strains and cells mutated in components of the classical nuclear protein import pathway were analyzed for the distribution of Ssa4p(1-236)-GFP under normal and stress conditions. (A) *SRP1* and *srp1-31* cells were grown at room temperature and incubated for 1 hour at 37°C to inactivate srp1-31p. Cells were subsequently incubated for 10 min with 10% ethanol and processed as described for Fig 1.1. Staining of DNA with DAPI and green fluorescence of Ssa4p(1-236)-GFP are shown. (B)–(D) Yeast strains carrying the alleles *GSP1, gsp1-2, RNA1, rna1-1, PRP20* or *prp20-1* were analyzed as described for (A).

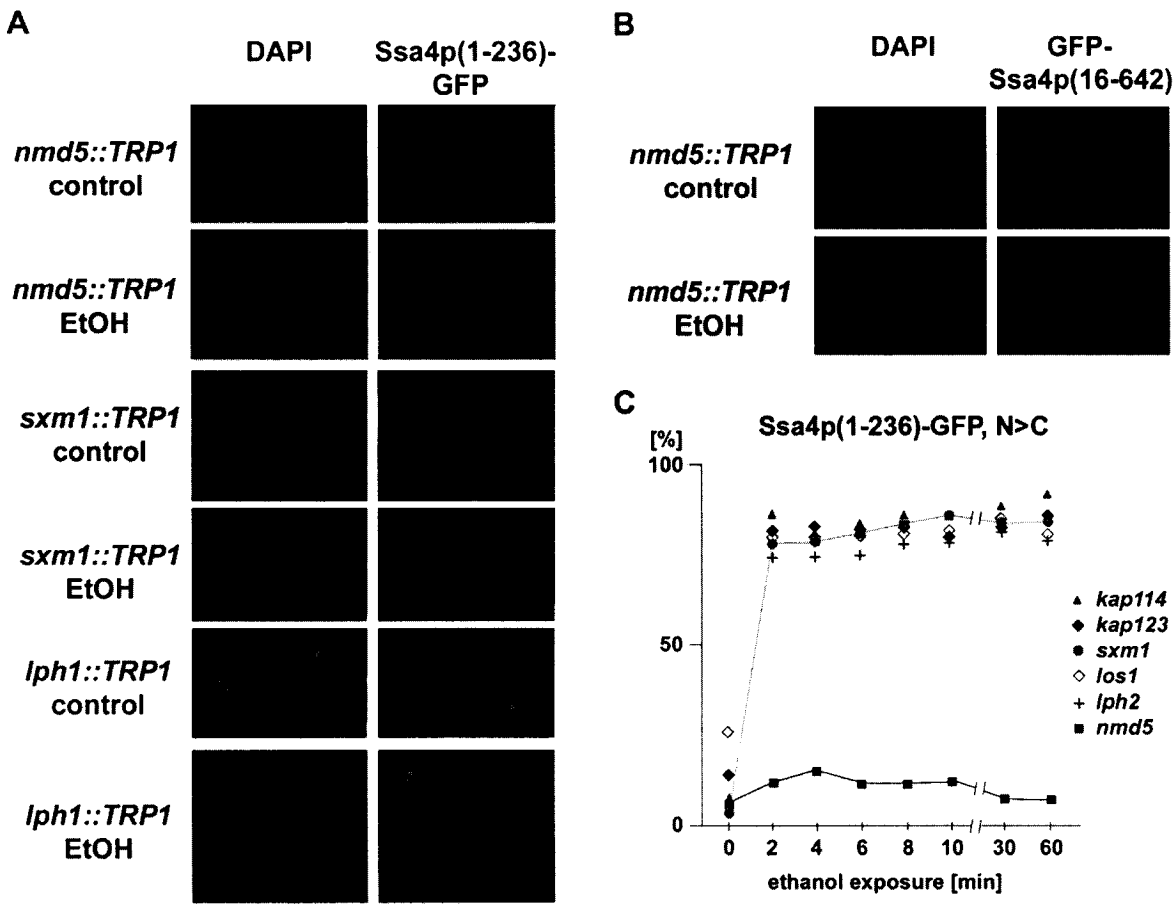

Figure 2.6 Ethanol-induced nuclear accumulation of Ssa4p(1-236) requires the carrier Nmd5p.

(A)Yeast strains carrying a mutation in one of the importin-β genes were analyzed for the nuclear accumulation of Ssa4p(1-236) in response to ethanol stress. The distribution of Ssa4p(1-236)-GFP is shown for strains *nmd5::TRP1, sxm1::TRP1 and lph2::TRP1.* Control and ethanol treated cells were analyzed in parallel. (B) GFP-Ssa4p(16-642) was located in *nmd5::TRP1* control and ethanol stressed cells as indicated. (C) Ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP in mutant yeast strains deleted for one of the importin-β genes. Mutant cells synthesizing Ssa4p(1-236)-GFP were stressed with 10% ethanol for the time indicated. Cells were fixed and nuclear accumulation of the fusion protein was monitored by fluorescence microscopy. Results were observed at least three times. Nuclear accumulation of Ssa4p(1-236)-GFP was determined at the times indicated, 100 cells were scored in each independent experiment for every time point and means are depicted in the figure.

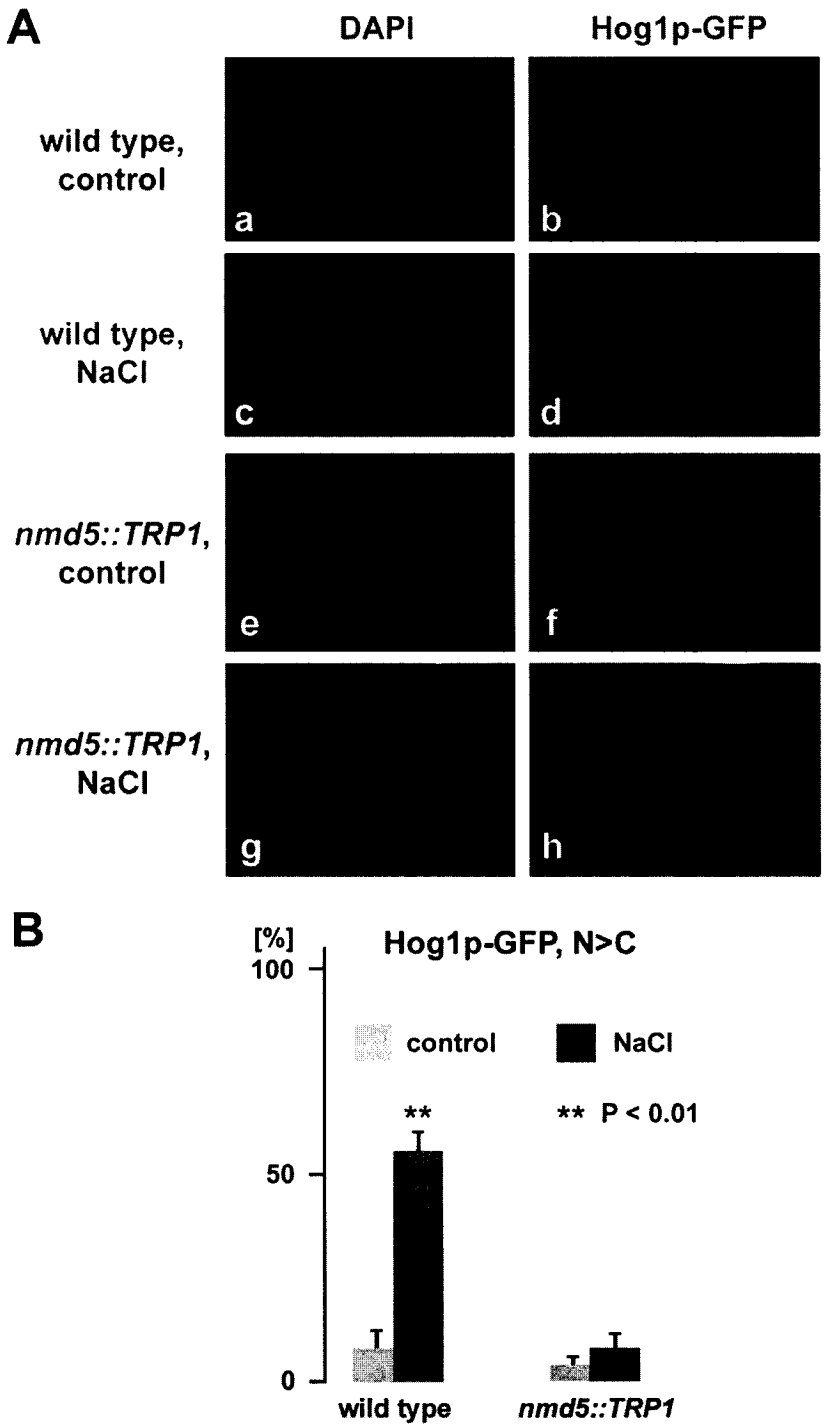

Figure 2.7 Nuclear accumulation of Hog1p-GFP in wild type and *nmd5::TRP1* cells.

(A) Nuclear accumulation of the reporter protein Hog1p-GFP was monitored in unstressed (control) cells and after a 10 min exposure to 0.4 M NaCl. (B) The accumulation of Hog1p-GFP (N>C) was monitored in at least 100 cells in each of three separate experiments. Means and S.D. are illustrated for wild type and *nmd5::TRP1* cells under nonstress and osmotic stress conditions.

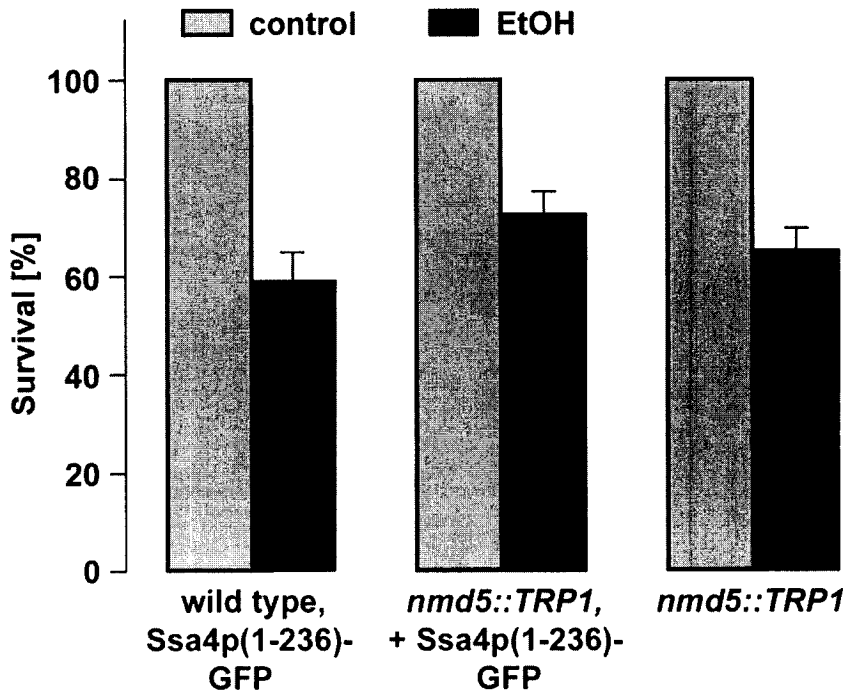

Figure 2.8 Viability of wild type and *nmd5::TRP1* cells after ethanol stress.

Wild type and *nmd5::TRP1* cells synthesizing Ssa4p(1-236)-GFP were treated for 10 min with 10% ethanol (EtOH) and immediately plated out.The formation of colonies was compared to unstressed cells.

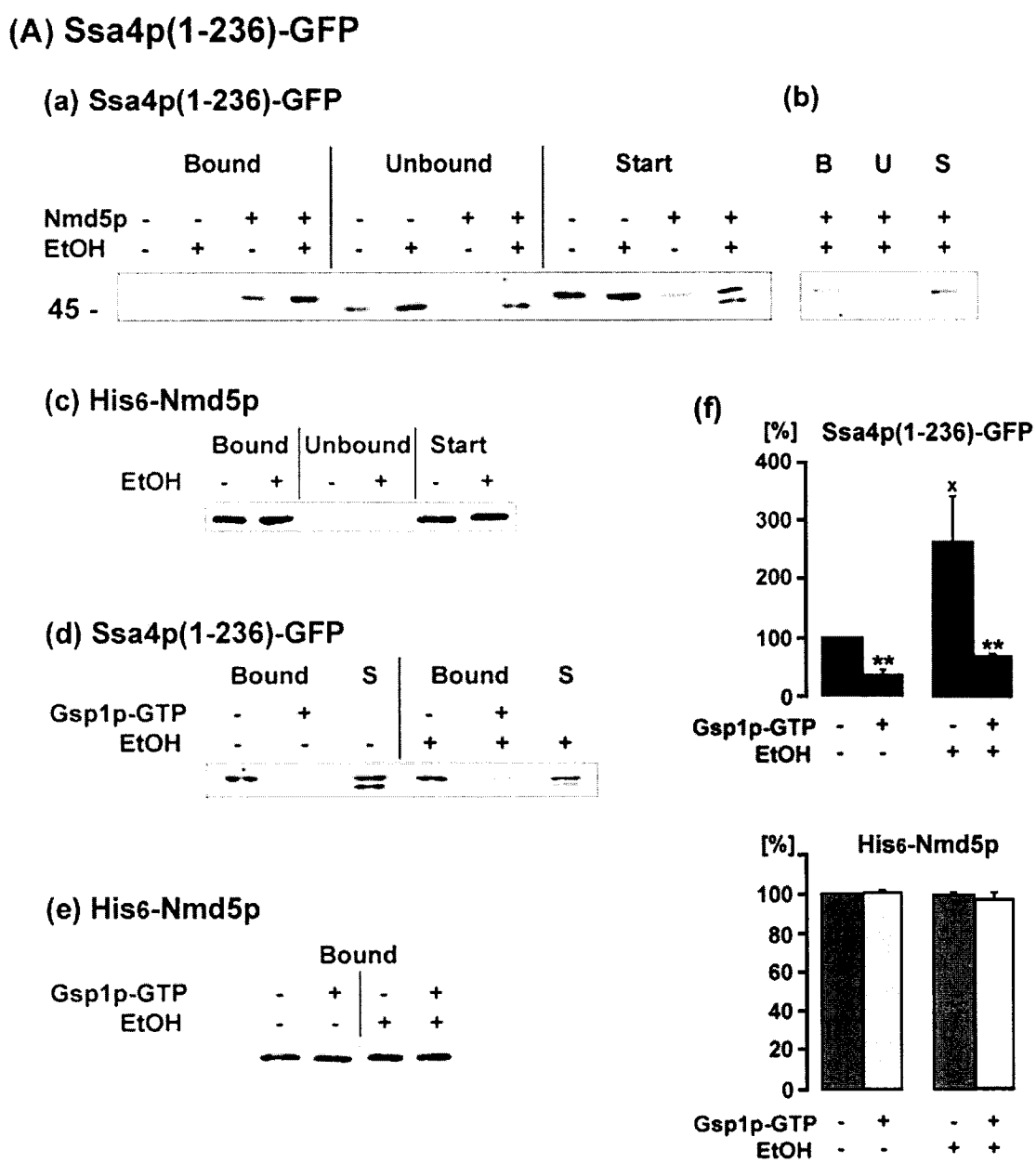

Figure 2.9 Binding of Ssa4p(1-236)-GFP to His6-tagged Nmd5p.

Figure 2.9 Binding of Ssa4p(1-236)-GFP to His6-tagged Nmd5p.

Part **A** of the figure shows the co-purification of Ssa4p(1-236)-GFP with His6-Nmd5p, part **B** control experiments carried out with the GFP-tag. Material bound to the metal affinity resin (B), 20% of the unbound proteins (U) and 20% of the starting material (S) were analyzed by Western Blotting with antibodies against GFP or the His6-tag. (**a**) Crude extracts were prepared from yeast cells synthesizing His6-Nmd5p, His6-Nmd5p and Ssa4p(1-236)-GFP or the GFP-tag as shown in the Figure. (**b**) Extracts were prepared from cells synthesizing His6-Nmd5p, Ssa4p(1-236)-GFP or GFP and mixed prior to affinity purification as indicated. (**c**) The purification of His6-Nmd5p was monitored under the same conditions as described for a. (**d**) The effect of GST-Gsp1pQ71L (Gsp1p-GTP) on the association of Ssa4p(1-236)-GFP with His6-Nmd5p was evaluated. Crude extracts from cells synthesizing Ssa4p(1-236)-GFP and His6-Nmd5p were incubated in the absence or presence of GST-Gsp1pQ71L (Materials and Methods) and protein complexes were purified by metal affinity chromatography. (**e**) The purification of His6-Nmd5p was followed for extracts from control and stressed cells, which were supplemented with GST-Gsp1pQ71L (Gsp1p-GTP) as indicated. Comparable amounts of His6-Nmd5p were isolated for different samples. (**f**) The co-purification of Ssa4p(1-236)-GFP with His6-Nmd5p was quantified for different experimental conditions by measuring the density of bands obtained after ECL (Materials and Methods). The association of Ssa4p(1-236)-GFP with His6-Nmd5 was determined for three independent experiments, binding of Ssa4p(1-236)-GFP to His6-Nmd5p in unstressed cells in the absence of GST-Gsp1pQ71L was defined as 100%. The interaction between Ssa4p(1-236)-GFP and His6-Nmd5p is drastically increased in ethanol stressed cells (x, $P = 0.04$). The formation of His6-Nmd5p/Ssa4p(1-236)-GFP complexes is significantly reduced by Gsp1p-GTP in control and stressed cells (**, $P < 0.01$). The purification of His6-Nmd5p was monitored under identical conditions. Similar amounts of the carrier were obtained, independent of stress or the presence of Gsp1p-GTP. Data shown are means and S.D. of three distinct experiments. Samples varied by a maximum of 3 +/- 3.7%, relative to the control (no Gsp1p-GTP, no ethanol), which was defined as 100%.

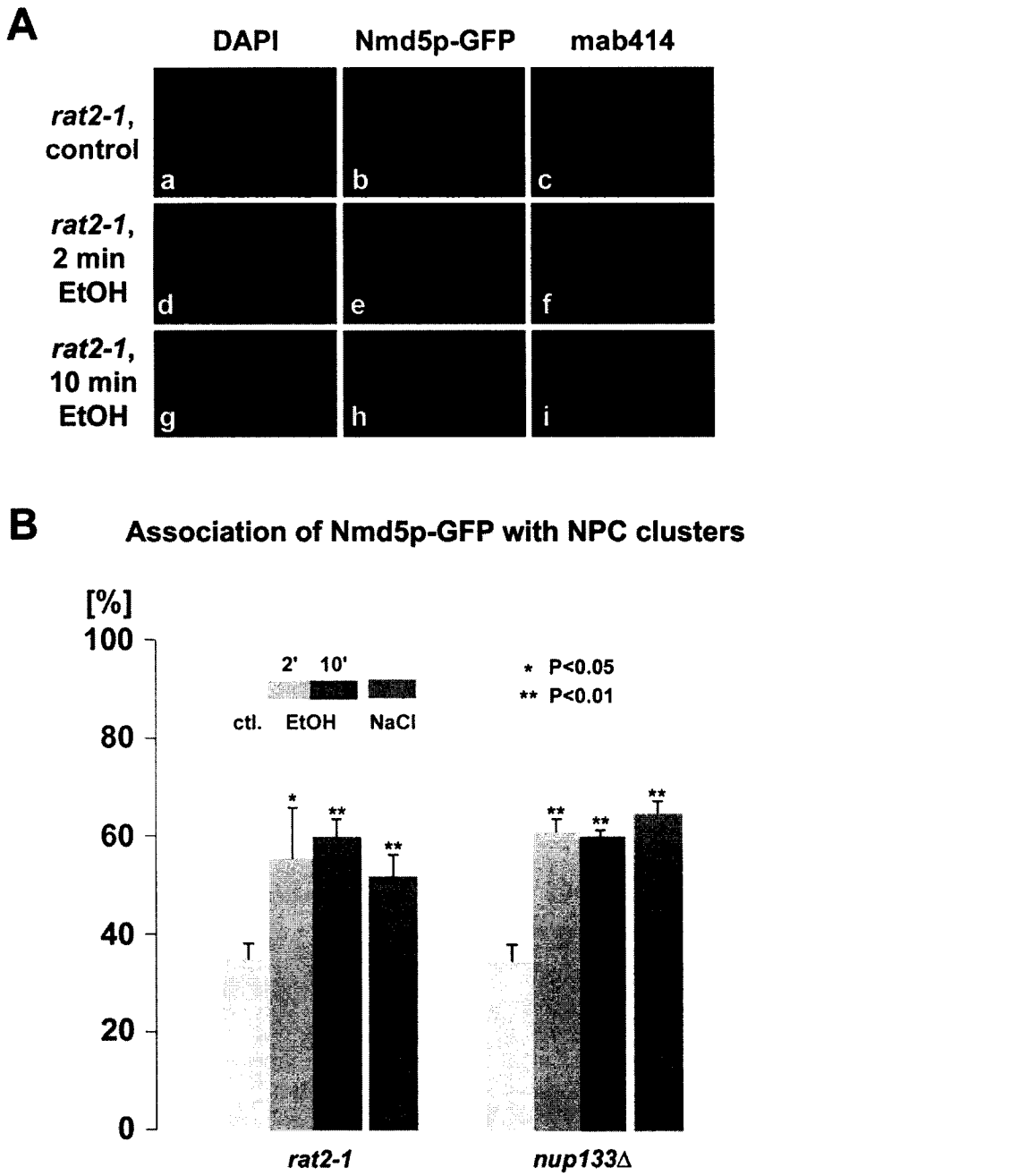

Figure 2.10 Nmd5p-GFP associates with NPCs in response to ethanol treatment.

NPC clustering strains *nup133Δ* and *rat2-1* were analyzed for the distribution of Nmd5p-GFP and NPC clusters under control and stress conditions, i.e. 2 min and 10 min 10% ethanol or 10 min 0.4 M NaCl. (**A**) Indirect immunofluorescence with antibodies against GFP and mab414 is shown for *rat2-1* cells. (**B**) Results obtained for *nup133Δ* and *rat2-1* were quantified. At least 100 cells were scored in each of three independent experiments carried out for the different experimental conditions depicted. Means and S.D. are shown.

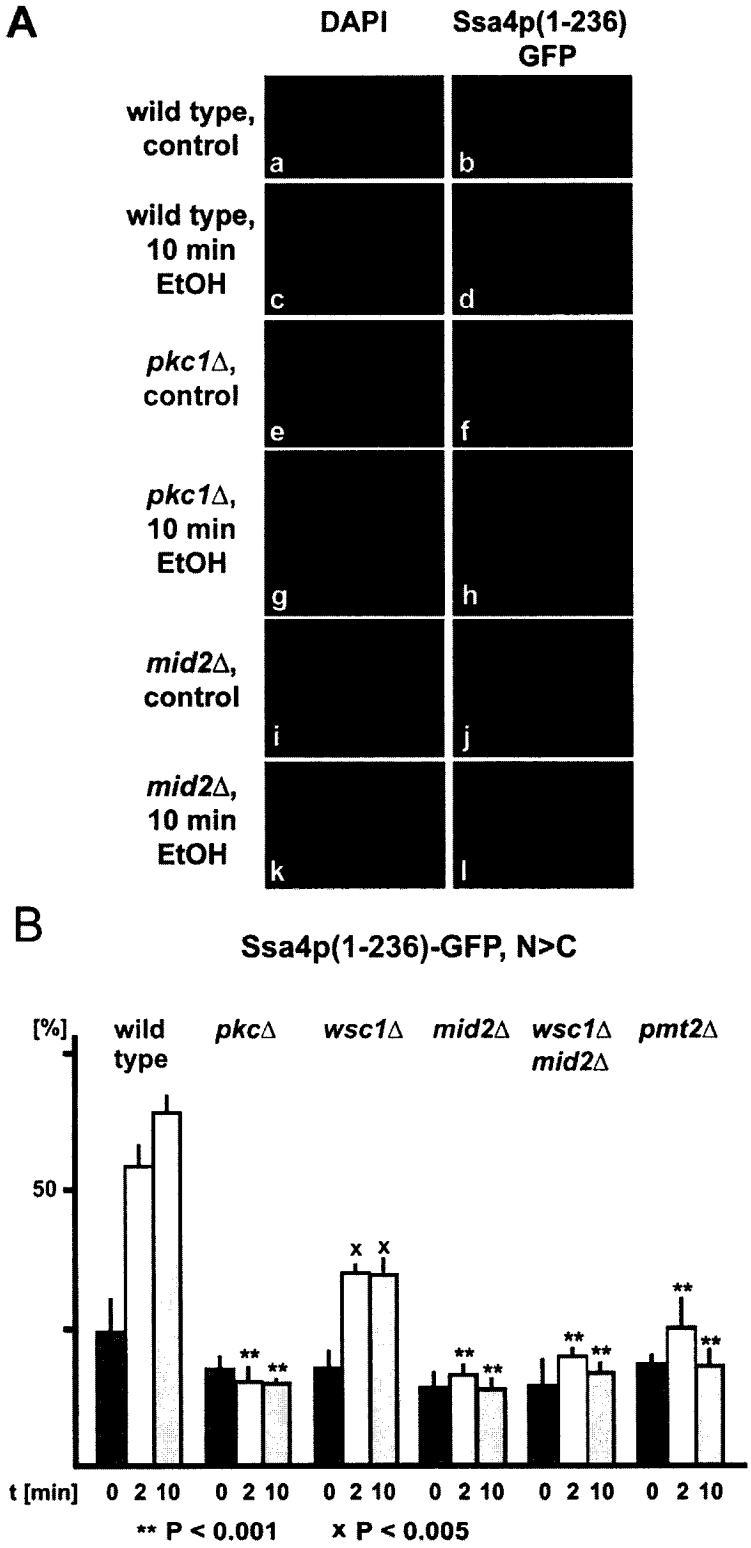

Figure 2.11 Nuclear accumulation of Ssa4p(1-236)-GFP in ethanol stressed cells requires components of the cell integrity pathway.

(**A**) The accumulation of Ssa4p(1-236)-GFP in nuclei of yeast strains lacking components involved in cell integrity signaling was analyzed under nonstress conditions (control) or after exposure to 10% ethanol for 10 min. (**B**) At least 100 cells were monitored in each of three separate experiments. Nuclear accumulation was scored in unstressed cells (0 min) and upon incubation with 10% ethanol for 2 or 10 min. Results obtained for mutants were compared to those of parental wild type cells. Means and S.D. are depicted for each strain tested (x, $P < 0.005$; **, $P < 0.001$).

CHAPTER 3

The carrier Msn5p/Kap142p promotes nuclear export of the hsp70 Ssa4p and relocates in response to stress

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3.1 Connecting text

In the previous chapter (chapter 2), the mechanisms of Ssa4p ethanol-induced unclear import is demonstrated. The concentration of Ssa4p in the nucleus of stressed cells is only transient. Upon removal of ethanol, the phenomena on Ssa4p relocation to the cytoplasm were described. In this chapter, we further studied the mechanisms of Sssa4p nuclear export during the recovery of stress. The exporting carrier Msn5p required for Ssa4p transfer back to the cytoplasm was identified. The export complexes of Ssa4p were detected both *in vivo* and *in vitro*. The role of a nucleoporin Nup82p and the Ran/Gsp1p system in Ssa4p export were analyzed. The regulation of stress on Msn5p-mediated Ssa4p nuclear export was studied. Furthermore, the effects of stress and carbon sources on carrier Msn5p localization were described in detail. This paper was published as ***Quan X,*** *Tsoulos P, Kuritzky A, Zhang R, Stochaj U. The carrier Msn5p/Kap142p promotes nuclear export of the hsp70 Ssa4p and relocates in response to stress. Mol Microbiol. 2006 Oct;62(2):592-609* (see Appendix).

3.2 Abstract

Cytoplasmic hsp70s like yeast Ssa4p shuttle between nucleus and cytoplasm under normal growth conditions but accumulate in nuclei upon stress. This nuclear accumulation is only transient, and Ssa4p relocates to the cytoplasm when cells recover. We show here that Ssa4p nuclear export is independent of Xpol/Crm1 and identify the importin-β family member Msn5p/Kap142p as the exporter for Ssa4p. In growing cells and in vitro, Msn5p and Ssa4p generate genuine export complexes that require Ran/Gsp1p-GTP. Furthermore, nucleoporin Nup82p, which plays a role in Msn5p-mediated transport, is necessary for efficient export of Ssa4p. In living cells, stress not only regulates Ssa4p localization, but also controls the distribution of Msn5p. Msn5p is concentrated in nuclei of unstressed cells, but appears in the cytoplasm upon exposure to ethanol, heat, starvation or severe oxidative stress. In addition, growth on non-fermentable carbon sources relocates a portion of Msn5p to the cytoplasm and leads to a partial nuclear accumulation of Ssa4p. Taken together, growth and stress conditions that localize the transporter Msn5p to the cytoplasm also induce the nuclear accumulation of its cargo Ssa4p.

3.3 Introduction

Cytoplasmic hsp70s shuttle in and out of the nucleus in higher eukaryotes as well as in the budding yeast Saccharomyces cerevisiae [Mandell, 1990; Quan, 2004; Kodiha, 2005]. As hsp70s are too large to translocate through the nuclear pore

complex (NPC) by diffusion, their transport across the nuclear envelope is likely to be an active process, which may depend on carrier proteins. In yeast, members of the Ssa and Ssb families of hsp70s are present in the cytoplasm, and Ssb1p is excluded from the nucleus due to the presence of a nuclear export signal (NES) that is recognized by Xpo1p [Shulga, 1999], the orthologue of metazoan Crm1 [reviewed in Ström, 2001]. In contrast to Ssb1p, Ssa proteins are located in both the cytoplasm and nuclei under non-stress conditions; they accumulate in nuclei when cells are exposed to various insults [Chughtai, 2001; Quan, 2004]. As such, the shuttling protein Ssa4p concentrates in nuclei when cells are exposed to ethanol, and we have identified the importin-β Nmd5p/Kap119p as the carrier that translocates Ssa4p into the nucleus of ethanol-treated cells [Quan, 2004]. At steady state, the localization of a shuttling protein can be controlled by different mechanisms, which may include changes in both nuclear import and export. Our previous studies have shown that in response to ethanol exposure Ssa4p nuclear import is upregulated on two different levels. First, the formation of import complexes containing Nmd5p and Ssa4p increases; second, docking of Nmd5p at the NPC is enhanced [Quan, 2004]. The subsequent concentration of Ssa4p in nuclei is only transient, and after removal of ethanol the chaperone relocates to the cytoplasm by an unknown mechanism.

Nuclear transport of most proteins relies on members of the importin-β family. In S. cerevisiae, 14 importin-β like carriers have been identified [reviewed in Wozniak, 1998; Ström, 2001; Mosammaparast, 2004] that translocate a large number of cargos through the NPC. Nuclear export mediated by these carriers

requires Ran/Gsp1p in its GTP-bound form, which associates with the carrier/cargo complex prior to its translocation across the nuclear envelope. Upon arrival in the cytoplasm Rna1p, the GTPase-activating protein for Gsp1p, stimulates the formation of Gsp1p-GDP, thereby promoting the disassembly of export complexes [reviewed in Weis, 2003; Mosammaparast, 2004].

In budding yeast, four members of the importin-β family participate in nuclear export. The carrier Xpo1p/Crm1 [Stade, 1997] moves a large number of proteins out of the nucleus, and NESs recognized by Xpo1p are characterized by the presence of hydrophobic amino acid residues, in particular leucine [reviewed in Kutay, 2005]. In higher eukaryotes, Crm1 is inhibited by the drug leptomycin B (LMB), a component that covalently modifies the carrier [Kudo, 1999]. Although Xpo1p in S. cerevisiae is not affected by LMB, a LMB-sensitive variant of Xpo1p has been generated that can functionally replace the wild-type protein [Neville, 1999]. Cse1p, another importin-β family member, exports selectively Srp1p, a subunit of the classical nuclear import receptor Srp1p/Kap95p [Hood, 1998; Solsbacher, 1998; Takano, 2005], whereas Los1p contributes to nuclear export of tRNA [Hellmuth, 1998; Sarkar, 1998; Steiner-Mosonyi, 2004]. Unlike Xpo1p, Cse1p and Los1p, the carrier Msn5p/Kap142p is not only involved in nuclear export, but also participates in protein import. Among the Msn5p export cargos identified so far are several proteins that play a role in signalling, cell cycle control and adaptation to changes in the growth medium [Alepuz, 1999]. For example, Msn5p exports Far1p, Pho4p, Crz1p, Ste5p, HO endonuclease and Mig1p, a protein involved in the repression of glucose-regulated genes [Kaffman,

1998; Blondel, 1999; DeVit, 1999; Mahanty, 1999; Boustany, 2002; Kaplun, 2003; Queralt, 2003]. Export of several Msn5p cargos is regulated by the growth conditions; in particular, the availability of nutrients like glucose and phosphate plays a role [reviewed in Hood, 1999]. In addition to export, Msn5p was reported to promote nuclear import of replication protein A [Yoshida, 2001]. In higher eukaryotes Msn5p-like carriers may have more complex functions, as the Arabidopsis Msn5p orthologue HASTY participates in the control of plant development [Bollman, 2003].

Although the effect of different physiological conditions on the localization of several Msn5p cargos has been characterized previously, it has yet to be determined whether changes in cell physiology also affect the distribution of the carrier. We show now that in *S. cerevisiae* Msn5p promotes nuclear export of the chaperone Ssa4p and that the localization of Msn5p is sensitive to stress and regulated by the carbon source. We propose that the distribution of Msn5p under different growth and stress conditions may contribute for some cargos to the regulation of Msn5p-mediated export.

3.4 Experimental procedures

3.4.1 Strains, plasmids and growth conditions

Yeast strains are shown in Table 3.1. Plasmids encoding Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) have been described previously [Quan, 2004]. For the experiments shown in Fig 3.1, DNA encoding GAL1 and SSA4(1-236)-GFP was

cloned into a centromeric plasmid carrying HIS3 as a selectable marker. Wild-type MSN5 was introduced with plasmid pEL335 [Alepuz, 1999], which is referred to as pMSN5. Alternatively, wild-type MSN5 was provided on a centromeric plasmid [Blondel, 1999; Fig 3.14). Cells were grown in rich (1% yeast extract, 2% peptone) or drop out medium containing 2% glucose, 2% galactose, 3% glycerol or 2% ethanol as a carbon source. Yeast transformation followed standard procedures; transformants were selected and grown on complete minimal medium lacking amino acids or nutrients used for selection.

3.4.2 Localization of GFP-fusion proteins and indirect immunofluorescence

Fusion proteins containing the GFP-tag were localized as described previously [Quan, 2004]. In brief, samples were analysed with a Nikon Optiphot at 1000× magnification. To avoid the non-linear range of fluorescence signals, cells highly overexpressing GFP or a GFP-tagged fusion protein were excluded from further analyses. Myc-tagged Msn5p inserted into the chromosome [Alepuz, 1999] was detected by indirect immunofluorescence with mab9E10 (Santa Cruz Biotechn., sc-40) essentially as in Gao et al. (2003). The localization of proteins was monitored by visual inspection of the specimens. At least 100 cells were scored in each of at least three independent experiments. The distribution of fluorescence was scored as described in Quan et al. (2004). N >> C denotes fluorescence can be detected in nuclei only, N > C, nuclear accumulation with weak signals detected in the cytoplasm; N + C, equal fluorescence signals are observed in nucleus and cytoplasm. Within every set of experiments, the same exposure time was used for all images. Negatives were scanned and processed with Adobe

Photoshop 8.0. Alternatively, images were taken with a Zeiss LSM 510 and processed in Adobe Photoshop 8.0.

3.4.3 Purification of tagged proteins from bacteria

GST- and His6-tagged proteins were synthesized in Escherichia coli. GST and GST-Gsp1p have been described previously [Quan, 2004]. A plasmid construct encoding GST-Msn5p was provided by G. Schlenstedt, His6-tagged versions of Rna1p, Ssa4p(1-236)-GFP and Ssa4(16-642) were generated by us using conventional methods. Proteins were purified under native conditions following standard procedures using glutathione Sepharose 4B (Amersham Biosciences, Baie d'Urfé, QC) or Ni-NTA agarose (Qiagen, Mississauga, ON).

3.4.4 Purification of Msn5p containing complexes

Protein complexes containing Myc-tagged Msn5p were isolated with Myc-specific antibodies under native conditions. In brief, cells synthesizing Myc-Msn5p and GFP-Ssa4p(16-642), Ssa4p(1-236)-GFP or GFP were grown overnight in selective medium containing 2% galactose to induce the synthesis of GFP-Ssa4p(16-642), Ssa4p(1-236)-GFP or GFP. Cells were grown to an OD600 of 1–1.3 and harvested by centrifugation. Sediments were stored at −70°C until use. All subsequent steps for lysis and immunoprecipitation were carried out at 4°C. The equivalent of 5 OD600 units was lysed with glass beads in buffer A [20 mM Pipes/KOH, 250 mM sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate, pH 7.5, 1% Triton X-100, 0.5 mM DTT, 1 mM PMSF, 5 mM benzamidine, and a protease inhibitor cocktail (antipain, aprotinin, chymostatin,

leupeptin and pepstatin; each at 1 µg ml1)]. Extracts were centrifuged for 5 min at 13 000 rpm (microfuge), and 1 ml of the supernatant was incubated with gentle agitation with 1 µg anti-Myc antibodies. After 2 h, 10 µl of protein G-Sepharose (Amersham Biotech.) equilibrated in buffer A was added and samples were incubated overnight. The resin was collected by centrifugation and washed three times with 250 µl buffer A. Material bound to the resin was released by boiling in gel sample buffer (10 min, 95°C) and supernatants (1 min, 13 000 rpm, microfuge) were analysed by Western blotting with antibodies against GFP [Quan, 2004]. To test the effect of the Gsp1p-GAP, His6-tagged Rna1p was purified from bacteria and added to 5 µM before the addition of anti-Myc antibodies.

3.4.5 Western blotting and quantization of ECL signals

Western blot analysis with antibodies against GFP, the His6- or Myc-epitope and quantization of signals was carried out as described [Quan, 2004]. In brief, 'Spot Density Tools' software was used to measure ECL signals as recommended by Alpha Innotech Corporation (San Leandro, CA). The density was determined for each area of interest and corrected for background. All of the samples measured for a particular experiment were present on the same filter. Films were exposed for different times to ensure that the brightest signal was below the saturation level of the film. The quantification was carried out for at least three independent experiments.

3.4.6 In vitro complex formation between Ssa4p and Msn5p

Ni-NTA resin (Qiagen) was equilibrated with PBS/KMT buffer (25 mM sodium phosphate, pH 7.3, 150 mM NaCl, 3 mM KCl, 1 mM MgCl2, 0.1% Tween 20, 1 mg ml1 BSA and a cocktail of protease inhibitors). One hundred and twenty microlitres of Ni-NTA resin was loaded with His6-Ssa4p(16-642) or Ssa4p(1-236)-GFP-His6 (each at 40 nM) for 1 h at 4°C and washed three times with 1 ml PBS/KMT buffer. The resin was divided into four identical aliquots and incubated in a final volume of 200 µl, containing 1 mM GTP and 40 nM GST-Msn5p, 200 nM GST-Gsp1pQ71L, 40 nM GST, alone or in combination (see Fig 3.4). Samples were incubated for 1 h at 4°C with gentle agitation and beads were washed three times in 1 ml PBS/KMT. Bound proteins were eluted in gel loading buffer (10 min, 95°C) and further analysed by SDS-PAGE and Western blotting with antibodies against GFP and GST.

3.5 Results

We have previously shown that the Ssa4p N-terminal portion and Ssa4p behave similar with respect to nuclear import [Quan, 2004]. Furthermore, Ssa4p contains a peptide binding domain in its C-terminal part, which is required to bind substrates that need folding/refolding. Interactions of the peptide binding domain with folding substrates can lead to retention within a cellular compartment, thereby preventing nucleocytoplasmic trafficking [Kodiha, 2005]. To analyse the role of carriers in Ssa4p nuclear transport, we were particularly interested in studying the N-terminal portion of Ssa4p.

3.5.1 The nuclear exporter Xpo1p/Crm1 is not essential for transport of Ssa4p to the cytoplasm

The carrier Xpo1p is involved in export of a large number of cargos, and NESs that bind Xpo1p are rich in hydrophobic amino acid residues, such as leucine [reviewed in Kutay, 2005]. A sequence matching the consensus of an Xpo1p-specific NES is present in the N-terminal domain of Ssa4p and we have tested the role of Xpo1p in export of Ssa4p(1-236)-GFP. To this end, two different strains that are mutated in *XPO1* were analysed (Fig 3.12, 3.13). We first monitored the distribution of Ssa4p(1-236)-GFP in a mutant yeast strain that carries a LMB-sensitive allele *XPO1 T539C* [Neville, 1999]. The fluorescent reporter protein NES-NLS-GFP2, which was used in control experiments, shuttles between nucleus and cytoplasm and is exported to the cytoplasm by Xpo1p [Stade, 1997]. In *XPO1 T539C* cells NES-NLS-GFP2 rapidly accumulates in nuclei upon addition of LMB [Neville, 1999 and Fig 3.12). By contrast, LMB failed to concentrate Ssa4p(1-236)-GFP in nuclei and no significant difference was detected when the localization of Ssa4p(1-236)-GFP in untreated controls, cells treated with solvent only or LMB was quantified (Fig 3.12).

In addition, a strain carrying the temperature-sensitive allele *xpo1-1* was exposed to 37°C for 15 or 30 min to inactivate xpo1-1p, and Ssa4p(1-236)-GFP was localized before and after heat treatment. Heat inactivation of xpo1-1p did not result in nuclear accumulation of the reporter protein, which was nuclear and cytoplasmic in *xpo1-1* and isogenic *XPO1* cells (Fig 3.13). After a 30 min exposure to 37°C, Ssa4p(1-236)-GFP was nuclear as well as cytoplasmic and in

part present in large aggregates in both mutant and wild-type cells. *xpo1-1* and *XPO1* cells showed similar localization of Ssa4p(1-236)-GFP after incubation at room temperature or 37°C. Taken together, these results are in line with the idea that Xpo1p does not play a major role in Ssa4p(1-236)-GFP nuclear export.

3.5.2 Ssa4p concentrates in nuclei of unstressed cells that contain a deletion of MSN5

We have previously localized Ssa4p(1-236)-GFP in different yeast strains that carry a deletion of one of the importin-β genes. These studies identified the carrier Nmd5p as an importer for Ssa4p(1-236)-GFP in ethanol-treated cells. Under non-stress conditions, all of the deletion mutants analysed so far located Ssa4p(1-236)-GFP to the nucleus and cytoplasm [Quan, 2004 and Fig 3.1]. Unlike other importin-β mutants, exemplified by *kap114Δ* in Fig. 1, *msn5Δ* had elevated levels of Ssa4p(1-236)-GFP in the nucleus even under normal growth conditions (Fig. 1A–C, arrow). Similar to the N-terminal 236 residues of Ssa4p, the fusion protein GFP-Ssa4p(16-642) accumulated also in the nucleus of unstressed *msn5Δ* cells (Fig. 1A and B). This mislocalization of Ssa4p(1-236)-GFP could be rescued by introduction of a wild-type copy of *MSN5* into *msn5Δ* cells. In cells that contained *MSN5* on an episomal plasmid (*pMSN5*), Ssa4p(1-236)-GFP was nuclear and cytoplasmic under non-stress conditions. Upon treatment with ethanol Ssa4p(1-236)-GFP accumulation in nuclei resembled wild-type cells (Fig 3.1A and B). Similar results were obtained when experiments were repeated with *MSN5* introduced into cells on a centromeric plasmid (Fig 3.14).

When *msn5Δ* cells were incubated with 10% ethanol, the number of cells that concentrated Ssa4p(1-236)-GFP in nuclei was further increased when compared with unstressed controls. The kinetics of ethanol-induced nuclear accumulation was similar in *msn5Δ*, wild-type cells or strains lacking other importin-β genes, and the maximum concentration in nuclei was reached after a 2 min ethanol treatment (Fig 3.1C). This increase of Ssa4p(1-236)-GFP nuclear accumulation in *msn5Δ* cells upon addition of ethanol is likely due to the upregulation of nuclear import [Quan, 2004], a process that is independent of the exporter *MSN5*.

The results for importin-β mutants described above are consistent with the idea that Msn5p participates in Ssa4p nuclear export and prompted us to further define the possible role of this carrier in Ssa4p nuclear trafficking.

3.5.3 Nuclear transport of Ssa4p(1-236)-GFP requires the nucleoporin Nup82p

The essential nucleoporin Nup82p is located at the cytoplasmic side of the NPC, where it participates in Msn5p-mediated export and other nuclear transport reactions [Belanger, 2004]. Moreover, Nup82p can directly interact with Msn5p [Damelin, 2000]. We therefore tested whether Nup82p is involved in Ssa4p(1-236)-GFP transport as well. Control and mutant cells were incubated for 1 h at 37°C, which will inactivate the temperature-sensitive nup82-3 protein. When compared with wild-type cells nup82-3 was less efficient in concentrating Ssa4p(1-236)-GFP in nuclei in response to ethanol exposure. This was seen both at room temperature and 37°C, but the defect was more severe in nup82-3 cells

that had been pre-incubated at the non-permissive temperature (Fig 3.2A). The addition of ethanol resulted in the reversible formation of aggregates for some cells, which disappeared after a 1 h recovery period (not shown).

To determine a possible contribution of nup82-3 to Ssa4p(1-236)-GFP export, all steps were carried out at the non-permissive temperature. Wild-type (W303) and mutant strains pre-incubated for 1 h at 37°C were treated for 10 min with 10% ethanol, and the percentage of cells that had accumulated the reporter protein in nuclei was given the arbitrary value of 1. Following ethanol exposure, cells were transferred to ethanol-free medium to monitor the relocation of Ssa4p(1-236)-GFP to the cytoplasm. When compared with wild-type cells treated under identical conditions, nup82-3 was less efficient in relocating Ssa4p(1-236)-GFP to the cytoplasm (Fig 3.2B). In addition to nup82-3, Ssa4p(1-236)-GFP nuclear export was tested in other strains that carry a mutation in a different nucleoporin gene. To this end, temperature-sensitive mutants nup1-8 and nsp1-5 were treated as described for nup82-3. Both nup1-8 and nsp1-5 were similar to wild-type cells with respect to nuclear export of Ssa4p(1-236)-GFP after recovery from ethanol stress (Fig 3.2B). In summary, these results suggest that Nup82p participates both in nuclear import and export of Ssa4p. The role of Nup82p in Ssa4p transport is specific as export inhibition was not detected for other nucleoporin mutations that were analyzed under the same conditions.

3.5.4 Ssa4p(1-236)-GFP generates export complexes with Msn5p in growing cells and *in vitro*

To characterize the role of Msn5p in nuclear transport of Ssa4p we tested whether both proteins can generate export complexes in living cells. Myc-tagged Msn5p integrated into the chromosome [Alepuz, 1999] was immunoprecipitated from crude cell extracts and purified material was probed with antibodies against GFP. Under these conditions, GFP-Ssa4p(16-642) and Ssa4p(1-236)-GFP, but not GFP, co-purified with Myc-Msn5p (Fig 3.3A). As Ssa4p(1-236)-GFP is missing the peptide-binding site of the hsp70, its association with Msn5p is unlikely to represent a simple chaperone/folding substrate interaction. Furthermore, the formation of nuclear export complexes containing members of the importin-β family requires Gsp1p-GTP, whereas chaperone/unfolded protein interactions do not depend on Gsp1p. The importance of Gsp1p-GTP was evaluated by adding the GTPase-activating protein Rna1p to crude extracts before purifying Msn5p/Ssa4p complexes. Stimulation of the Gsp1p-GTPase activity by Rna1p will lower the concentration of Gsp1p-GTP, thereby interfering with export complex formation. As shown in Fig 3.3A, the addition of Rna1p drastically reduced the amount of GFP-Ssa4p(16-642) or Ssa4p(1-236)-GFP that co-purified with the carrier, supporting the idea that Gsp1p-GTP stimulates the association of Msn5p and Ssa4p.

In order to determine whether Ssa4p and Msn5p can interact directly, binding was tested with purified components in vitro using immobilized metal affinity chromatography and His6-tagged Ssa4p proteins as a bait. In the absence of Gsp1p-GTP we detected only little association of Ssa4p(1-236)-GFP with the carrier Msn5p (Fig 3.3B). However, this interaction was increased to 5.5-fold by

the addition of Gsp1p-GTP (Fig 3.3B). In control experiments, non-specific binding of Msn5p or GST to the resin was negligible and Gsp1p-GDP had no effect on the formation of transport complexes. We further tested His6-tagged Ssa4p in pull-down experiments under identical conditions (Fig 3.3C). Like the N-terminal portion of Ssa4p, His6-Ssa4p(16-642) binds Msn5p *in vitro*, and the interaction is stimulated to 5.7-fold binding in the presence of Gsp1p-GTP. Thus, both proteins generate genuine nuclear export complexes with Msn5p in growing cells and *in vitro*.

3.5.5 Msn5p localization is regulated by the exposure to stress, but independent of the presence of cargo

Several stressors promote the nuclear accumulation of Ssa4p, whereas exposure to osmolytes or mild oxidative stress is not efficient in concentrating the chaperone in nuclei [Chughtai, 2001; Quan, 2004]. Like ethanol treatment or starvation [Chughtai, 2001; Quan, 2004], we have identified heat shock (6 h at 37°C or 1 h at 42°C) as another form of stress that accumulates Ssa4p(1-236)-GFP in nuclei (Fig 3.4C). To determine whether environmental changes that redistribute Ssa4p(1-236)-GFP also affect Msn5p, the carrier was localized in control and stressed cells. Msn5p accumulated in nuclei under control conditions, but appeared in the cytoplasm in response to ethanol, heat, starvation or severe oxidative stress (Fig 3.4A, N + C). When different types of stress were compared, starvation was the most effective and all of the starving cells redistributed Msn5p. By contrast, exposure to osmotic or mild oxidative stress had no severe effects. Likewise, these treatments do not alter drastically the localization of Ssa4p [Quan,

2004]. The stress-induced changes in the distribution of Msn5p do not simply reflect degradation of the carrier. Western blot analysis of crude extracts prepared from control and treated cells revealed that the levels of Msn5p were similar under all conditions tested (Fig 3.4B and data not shown).

One possible explanation for the concentration of Msn5p in unstressed cells could be a retention process whereby the carrier binds to cargo in the nucleus. For instance, cargo associated with chromatin or other nuclear structures might prevent the carrier from moving to the cytoplasm. A mislocalization of Msn5p could then be explained by the absence of cargo from the nucleus. If this was the case, one would predict that a reduction of cargo in nuclei will lead to an increase of cytoplasmic Msn5p levels. To begin to address this question, we compared the localization of Msn5p-GFP in wild-type cells and mutants *pho4Δ* or *far1Δ* (Fig 3.4D). Msn5p-GFP was concentrated in nuclei for all strains tested, and no drastic changes were detected in mutant cells. This suggests that the deletion of either cargo does not alter Msn5p distribution; however, we cannot rule out that other cargos might contribute to the nuclear retention of Msn5p.

3.5.6 Stress controls the formation of Msn5p export complexes

In addition to the mislocalization of Msn5p to the cytoplasm, other mechanisms may prevent Ssa4p nuclear export upon stress. One level of regulation could be the formation of export complexes containing Msn5p and Ssa4p(1-236)-GFP. To test this hypothesis, pull-down experiments were carried out with crude extracts from cells that had been exposed to different stresses (Fig 3.5). Exposure of cells

to heat, starvation or ethanol drastically reduced the association between carrier and cargo, supporting the idea that these conditions reduce the formation of export complexes.

3.5.7 Ethanol-induced relocation of Msn5p is reversible during stress recovery

Nuclear accumulation of the Msn5p-cargo Ssa4p(1-236)-GFP in ethanol-treated cells is reversible, and Ssa4p(1-236)-GFP migrates back to the cytoplasm when ethanol is removed. This export to the cytoplasm is independent of de novo protein synthesis [Quan, 2004]. To analyse whether the Ssa4p exporter Msn5p also redistributes during recovery, cells were treated for 10 min with 10% ethanol and subsequently transferred to ethanol-free medium. Removal of ethanol induced the re-accumulation of Msn5p in nuclei. This process does not require protein synthesis, as it was insensitive to cycloheximide (Fig 3.6A and B). The kinetics of Msn5p and Ssa4p(1-236)-GFP relocation during stress recovery suggests that increasing the concentration of Msn5p in nuclei correlates with the appearance of Ssa4p(1-236)-GFP in the cytoplasm (Fig 3.6A and B).

3.5.8 Msn5p and Ssa4p(1-236)-GFP localization is regulated by the carbon source

Msn5p is involved in nuclear export of proteins that regulate glucose-dependent gene expression, such as Mig1p, and we tested whether growth on fermentable versus non-fermentable carbon sources affects the localization of Msn5p. As reported previously [Alepuz, 1999], the carrier is concentrated in nuclei of cells

growing in rich medium supplemented with glucose. However, when cells were grown on YP-medium containing glycerol or ethanol approximately half of the cells showed Msn5p in the cytoplasm (data not shown). Similar results were obtained when minimal medium was used instead of YP (Fig 3.7A). Moreover, like Msn5p the distribution of Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) were also regulated by the carbon source, and increased amounts of the reporter protein were detected in nuclei whenever elevated levels of Msn5p were in the cytoplasm (Fig 3.7A).

In control experiments, the Mig1p transport domain (residues 261–400; DeVit, 1997) fused to GFP-β-galactosidase, here referred to as Mig1p-GFP-β-gal, was analysed (Fig 3.7A). Mig1p controls the expression of glucose-regulated genes, and nucleocytoplasmic trafficking of Mig1p mediated by the transport domain is crucial to this process [DeVit, 1997; DeVit, 1999]. Msn5p is the nuclear exporter for Mig1p, and the nuclear accumulation of Mig1p depends on the availability of glucose [DeVit, 1997]. As expected, in glucose-containing medium, Mig1p-GFP-β-gal is restricted to nuclei, but mostly cytoplasmic when cells were provided with 3% glycerol or 2% ethanol as a carbon source.

To test in more detail how a shift from fermentable to non-fermentable carbon source affects Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) localization, cells were grown overnight in minimal medium containing 2% galactose and transferred to medium containing either glycerol or ethanol (Fig 3.7B). After 1 h incubation in medium containing glycerol or ethanol, nuclear concentration of Ssa4p(1-236)-

GFP and GFP-Ssa4p(16-642) was observed in 77–79% and 71–73% of the cells respectively. After continued growth on glycerol or ethanol nuclear accumulation was maintained at a level of 54–58% for Ssa4p(1-236)-GFP and 48–49% for GFP-Ssa4p(16-642).

3.5.9 Different mechanisms control nuclear export of the transcription factor Mig1p and the hsp70 Ssa4p

Additional control studies were carried out to determine whether stress alters in general the distribution of Msn5p-cargos. To this end, the localization of Mig1p-GFP-β-gal, Pho4p-GFP and Far1p-GFP was monitored in cells exposed to heat, ethanol for 10 min, starvation, osmotic or oxidative stress (Fig 3.8 and 3.9). Starvation was very efficient in relocating Mig1p-GFP-β-gal to the cytoplasm, whereas none of the other treatments prevented nuclear accumulation of Mig1p-GFP-β-gal. Although some of the cells exposed to 10% ethanol showed faint cytoplasmic staining, most of Mig1p-GFP-β-gal remained concentrated in nuclei in response to 10 min ethanol stress (Fig 3.8). Unlike Mig1p, Pho4p-GFP and Far1p-GFP were efficiently relocated to the cytoplasm upon exposure to heat, starvation and ethanol (Fig 3.9).

In summary, the distribution of Ssa4p(1-236)-GFP correlated with the location of Msn5p; whenever Msn5p was detected in the cytoplasm, elevated levels of Ssa4p(1-236)-GFP appeared in the nucleus. No such link could be detected for Msn5p and Mig1p-GFP-β-gal, whereas Pho1p-GFP and Far1p-GFP were also

mislocalized in response to heat, starvation and ethanol. In all cases, starvation had the strongest effect on relocating Msn5p and its cargos.

3.5.10 Mutant *msn5Δ* and *nmd5Δ* cells are more sensitive to stress than wild-type cells

Although *MSN5* and *NMD5* are not essential genes it was possible that either carrier improves the survival of stressed cells. We therefore tested the viability of strains lacking *MSN5* or *NMD5* after exposure to different stresses and compared them with the wild-type strain W303, which has the same genetic background as both mutants (Fig 3.10). Survival rates were measured as the number of colonies formed on plates, and survival of W303 was defined as 100% for each of the stress conditions tested. For heat, starvation, ethanol and 1 h exposure to oxidative stress *msn5Δ* and *nmd5Δ* had lower survival rates than wild-type cells, suggesting an important role of either carrier in the proper response to some forms of stress.

3.5.11 Ssa4p protects yeast cells from stress

To evaluate the possible contribution of Ssa4p to stress survival, yeast cells that carry the *SSA1* gene as the only member of the *SSA* family (JN516; Becker, 1996) were exposed to heat, starvation, ethanol, osmolytes and oxidants. The survival rates were compared with cells that, in addition to Ssa1p, synthesize GFP-Ssa4p(16-642) or Ssa4p(1-236)-GFP. Cells with the same genetic background but containing all members of the *SSA* family (*SSA1 SSA2 SSA3 SSA4*; JN55; Becker, 1996) were used as a wild-type control and the survival of this strain was defined as 100% for each stress analysed. In the absence of *SSA2 SSA3 SSA4* cells were

severely affected by stress (Fig 3.11); however, overexpression of GFP-Ssa4p(16-642) could rescue this strain and survival was similar to the wild-type control. Some protection against stress was also observed for Ssa4p(1-236)-GFP, but it was always less than for GFP-Ssa4p(16-642). These results are consistent with the idea that the synthesis of Ssa4p helps to protect cells from stress-induced death.

3.6 Discussion

Our studies were undertaken to identify and characterize the nuclear exporter for Ssa4p, a chaperone that shuttles between nucleus and cytoplasm. Ssa4p concentrates in nuclei in response to certain environmental changes, and we have previously shown that the importin-β Nmd5p is required for Ssa4p nuclear accumulation in cells that have been exposed to ethanol or starvation [Chughtai, 2001; Quan, 2004]. Specifically, ethanol treatment upregulates nuclear import of Ssa4p(1-236)-GFP by stimulating the formation of Ssa4p(1-236)-GFP/Nmd5p import complexes and docking of Nmd5p at the NPC [Quan, 2004].

The results presented here show that Msn5p, a carrier that participates both in nuclear import and export of proteins, translocates Ssa4p from nuclei to the cytoplasm. The steady-state distribution of a shuttling protein is the result of nuclear import, export and retention in either cellular compartment. So far, it is not known whether retention contributes to the intracellular localization of Ssa4p. We have therefore focused on the mechanisms that are involved in Ssa4p transport across the nuclear envelope. Our mutant analyses show that even under

non-stress conditions Ssa4p is accumulated in nuclei of *msn5Δ* cells, suggesting that impaired export of the hsp70 increases its nuclear concentration. Moreover, when a wild-type copy of *MSN5* is introduced into the *msn5Δ* mutant Ssa4p redistributes throughout nucleus and cytoplasm in unstressed cells. Thus, we conclude that nuclear export promoted by Msn5p plays a role in the regulation of Ssa4p distribution. Further support for this hypothesis comes from the analysis of nup82-3, a mutant nucleoporin that affects Msn5p-mediated export [Belanger, 2004]. Surprisingly, nup82-3 cells have an additional defect in ethanol-induced Ssa4p nuclear import. Nup82p does not only participate in Msn5p-dependent trafficking, this nucleoporin is also involved in other nuclear transport pathways, such as classical nuclear import and interacts with the importin-β-like carrier Pse1p [Damelin, 2000; Belanger, 2004]. Based on these data, a possible explanation for the impaired Ssa4p import in nup82-3 cells could be the direct or indirect interaction of Nup82p with Nmd5p, the carrier that translocates Ssa4p into nuclei of ethanol-treated cells [Quan, 2004]. A direct role of Msn5p in Ssa4p nuclear export was demonstrated here by the purification of transport complexes from growing cells that contain both the carrier and hsp70. Importantly, the interaction was reduced by the Gsp1p-GTPase-activating protein Rna1p, suggesting that Msn5p and Ssa4p are components of a genuine nuclear export complex that is comprised of carrier/cargo/Gsp1p-GTP. This model is consistent with in vitro binding assays, which show that a direct interaction between Msn5p and Ssa4p is stimulated significantly by Gsp1p-GTP but not Gsp1p-GDP. Taken together, the results for growing cells as well as *in vitro* data demonstrate an essential role for Msn5p in Ssa4p nuclear export. Unlike Ssa4p, other cytoplasmic

hsp70s may use distinct nuclear carriers for export to the cytoplasm. This is exemplified by Ssb1p, which is excluded from nuclei under steady-state conditions due to its export by Xpo1p [Shulga, 1999].

MSN5 or *NMD5* are not essential genes, yeast cells lacking a functional copy of either gene display a lower viability than wild-type strain W303, which has the same genetic background as the carrier mutants. Although Ssa4p is one of the cargos whose nuclear transport is altered by *msn5Δ* and *nmd5Δ*, it is obvious that the absence of Msn5p or Nmd5p will impact a larger number of proteins that move in and out of the nucleus. For instance, the distribution of Msn2p and Hog1p, proteins involved in the response to oxidative and osmotic stress, is regulated by Msn5p and Nmd5p respectively [Ferrigno, 1998; Görner, 2002]. The lack of proper trafficking of cargos other than Ssa4p, which are required for the appropriate response and adaptation to stress, is likely to contribute to the reduced survival of *msn5Δ* and *nmd5Δ* cells. Like Msn5p and Nmd5p, Ssa4p can also affect the survival of stress. Yeast cells carrying *SSA1* as the only gene of the *SSA* family are sensitive to a variety of treatments, but overexpression of *SSA4* can drastically increase survival rates when these cells are stressed. Surprisingly, when compared with cells synthesizing Ssa1p only, expression of *SSA4(1-236)-GFP* also somewhat improved stress survival, but in contrast to the expression of *GFP-SSA4(16-642)* the differences were not statistically significant. The fact that GFP-Ssa4p(16-642), but not Ssa4p(1-236)-GFP, improved survival of stressed cells suggests that the C-terminal peptide binding domain participates in the

protection against stress-induced damage. These data are consistent with the idea that the chaperone activity of Ssa4p contributes to the increase in stress survival.

Our studies show that nuclear export mediated by Msn5p is crucial for the regulation of Ssa4p distribution under non-stress conditions. Moreover, not only the cargo Ssa4p, but also the localization of its carrier Msn5p is controlled by the physiological state of the cell. As such, Msn5p localization is sensitive to stress exposure as well as the composition of the growth medium. In particular, growth on non-fermentable carbon sources, ethanol, heat, starvation or severe oxidative stress induces a redistribution of Msn5p from nuclei to the cytoplasm. To our knowledge, these results are the first studies to report how different stress and growth conditions regulate the localization of a nuclear transporter. Interestingly, the presence of Msn5p in the cytoplasm was found to correlate with the nuclear accumulation of Ssa4p. It is possible that the cytoplasmic localization of Msn5p may limit the trafficking across the nuclear envelope and play a role in the regulation of specific nuclear import and export processes. Moreover, stress conditions that relocate Msn5p to the cytoplasm also inhibit the complex formation between Ssa4p(1-236)-GFP and Msn5p. It is tempting to speculate that Msn5p under these conditions is unable to interact with export cargo. For instance, it is possible that post-translational modifications of carrier and/or cargo in stressed cells prevent the interaction between Msn5p and Ssa4p(1-236)-GFP, and this question will have to be addressed in future studies.

We do not expect that the availability of Msn5p in the nucleus is the limiting factor that controls nucleocytoplasmic trafficking for all of its cargos. For example, the transcription factors Mig1p and Pho4p display a complex transport regulation which requires their modification for Msn5p-dependent translocation to the cytoplasm [Ferrigno, 1998; Kaffman, 1998; reviewed in Hood, 1999]. Our data for Mig1p show clearly that Msn5p distribution is not the critical reaction for localizing this transcription factor. The relative abundance of a particular cargo and the affinity for the carrier may also play a role in its steady-state distribution. As such, Ssa4p is more than 22 times as abundant when compared with Mig1p and about five times as abundant as Msn5p [Ghaemmaghami, 2003]. Moreover, in the case of Mig1p and Pho4p binding to Msn5p is controlled by phosphorylation. Thus, competition between different proteins for binding to the carrier can add another layer of complexity that controls the localization of individual cargos.

In summary, we have shown a correlation between the localization of Msn5p in the cytoplasm and Ssa4p in the nucleus. Whenever elevated levels of Msn5p are present in the cytoplasm increased concentrations of Ssa4p are detected in the nucleus. On the basis of this observation, it is tempting to speculate that the steady-state distribution of Msn5p may contribute to the export efficiency of some cargos. It is conceivable that regulating the levels of Msn5p in the nuclear or cytoplasmic compartment could provide an additional tool to adjust nuclear trafficking of selected cargos to the needs dictated by the physiological state of the cell.

3.7 Acknowledgements

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Table 3.1 Yeast strains used in this study.

Strain	Genotype	Reference
W303	*Mata ade2 his3 leu2 trp1 ura3*	
msn5Δ	*Matα ade2his3 leu2 trp1 ura3 can1 msn5::Tn LEU2*	Chughtai et al., 2001
MSN5-MYC	*ade2 his3 leu2 ura3 trp1 can1 MSN5-MYC*	Alepuz et al., 1999
kap114Δ	*his3 leu2 ura3 trp1 kap114::TRP1*	Chughtai et al., 2001
xpo1-1	*Matα ade2 his3 leu2 ura3 trp1 xpo1::LEU2* (pKW-*HIS3-xpo1-1*)	Stade et al., 1997
XPO1	*Matα ade2 his3 leu2 ura3 trp1 xpo1::LEU2* (pKW-*HIS3-XPO1*)	Stade et al., 1997
XPO1 T539C	*ura3 XPO1/CRM1 T539C*	Neville and Rosbash, 1999
nup82-3	*Matα ade2 his3 leu2 ura3 trp1*	Belanger et al., 2004
NUP1	*Matα his3 leu2 trp1 ura3 nup1::LEU2* (*NUP1-TRP-CEN)*	
nup1-8	*Matα his3 leu2 trp1 ura3 nup1::LEU2* (*nup1-8-TRP-CEN*)	
nsp1-5	*Matα ade 2 his3 leu2 trp1 ura3 nsp1::HIS3* (*pSB32-LEU2-nsp1-5*)	Nehrbass et al., 1990
JN55	*his3 leu2 lys2 trp1 ura 3 SSA1 SSA2 SSA3 SSA4*	Becker et al., 1996
JN516	*his3 leu2 trp1 ura 3 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2 SSA1*	Becker et al., 1996

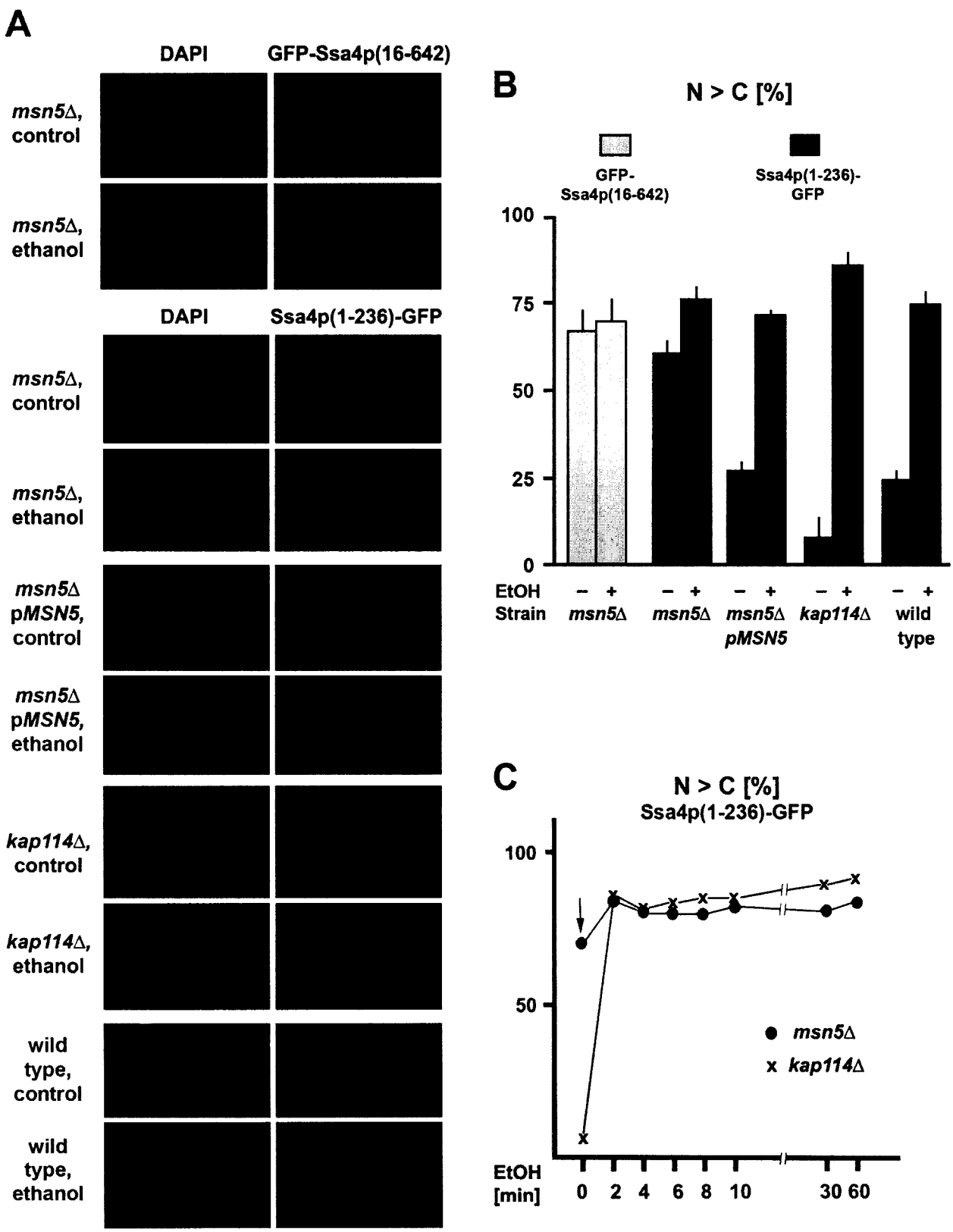

Fig 3.1 Nuclear export of Ssa4p is mediated by the importin-β Msn5p.

Fig 3.1 Nuclear export of Ssa4p is mediated by the importin-β Msn5p.

(**A**) Yeast strain *msn5Δ* was analyzed for the distribution of GFP-Ssa4p(16-642) or Ssa4p(1-236)-GFP under non-stress conditions and upon addition of ethanol. Wild type *MSN5* encoded by an episomal plasmid (p*MSN5*) was introduced into strain *msn5*□followed by localization of Ssa4p(1-236)-GFP. For comparison, the importin-β mutant *kap114Δ* and wild type strain W303 were analyzed under identical conditions. (**B**) The localization of fluorescent reporter proteins was determined in at least 100 cells. Means and standard deviations are shown for at least three independent experiments. (**C**) Nuclear accumulation of Ssa4p(1-236)-GFP was monitored at different time points in *msn5Δ* and *kap114Δ* cells. Note that under non-stress conditions at 0 min elevated levels of Ssa4p(1-236)-GFP are present in nuclei (indicated by an arrow).

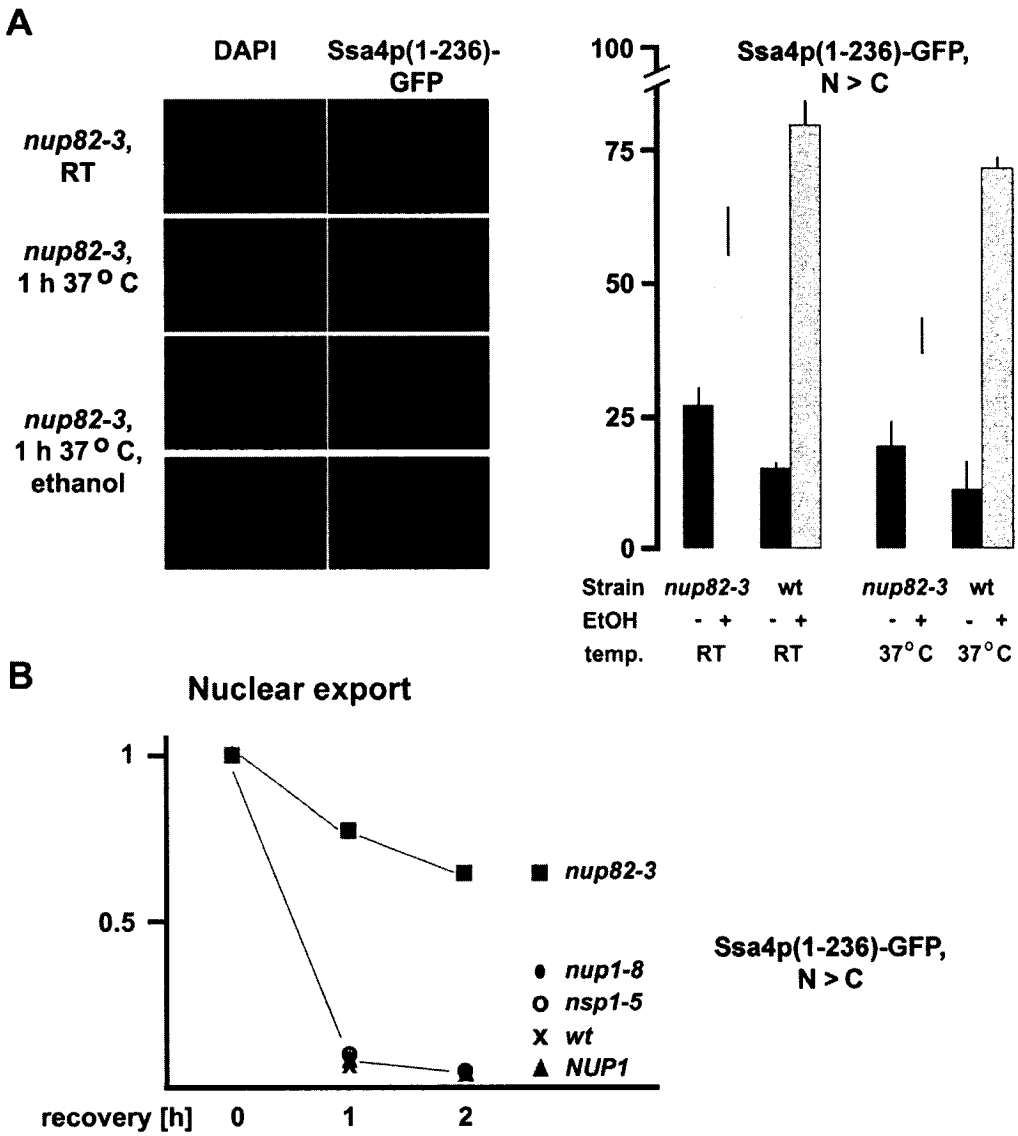

Fig 3.2 The nucleoporin Nup82p is involved in nuclear transport of Ssa4p(1-236)-GFP.

Fig 3.2 The nucleoporin Nup82p is involved in nuclear transport of Ssa4p(1-236)-GFP.

(**A**) Wild type and nup82-3 cells were kept at room temperature (RT) or incubated for 1 h at 37°C and subsequently treated for 10 min with 10% ethanol. Ssa4p(1-236)-GFP was localized by fluorescence microscopy. The distribution of Ssa4p(1-236)-GFP was quantified as in Fig. 1. (**B**) Export of Ssa4p(1-236)-GFP to the cytoplasm is compromised in nup82-3 mutants at the non-permissive temperature. The number of wild type or mutant cells, respectively, that show nuclear accumulation of Ssa4p(1-236)-GFP after ethanol treatment was defined as 1. After removal of ethanol, aliquots of the cultures were fixed and the distribution of Ssa4p(1-236)-GFP was quantified. For comparison, nucleoporin mutants nup1-8 and nsp1-5 and wild type strains (W303, wt; NUP1) were analyzed under the same conditions. Data are shown for three independent experiments; at least 100 cells were scored for each data point and experiment.

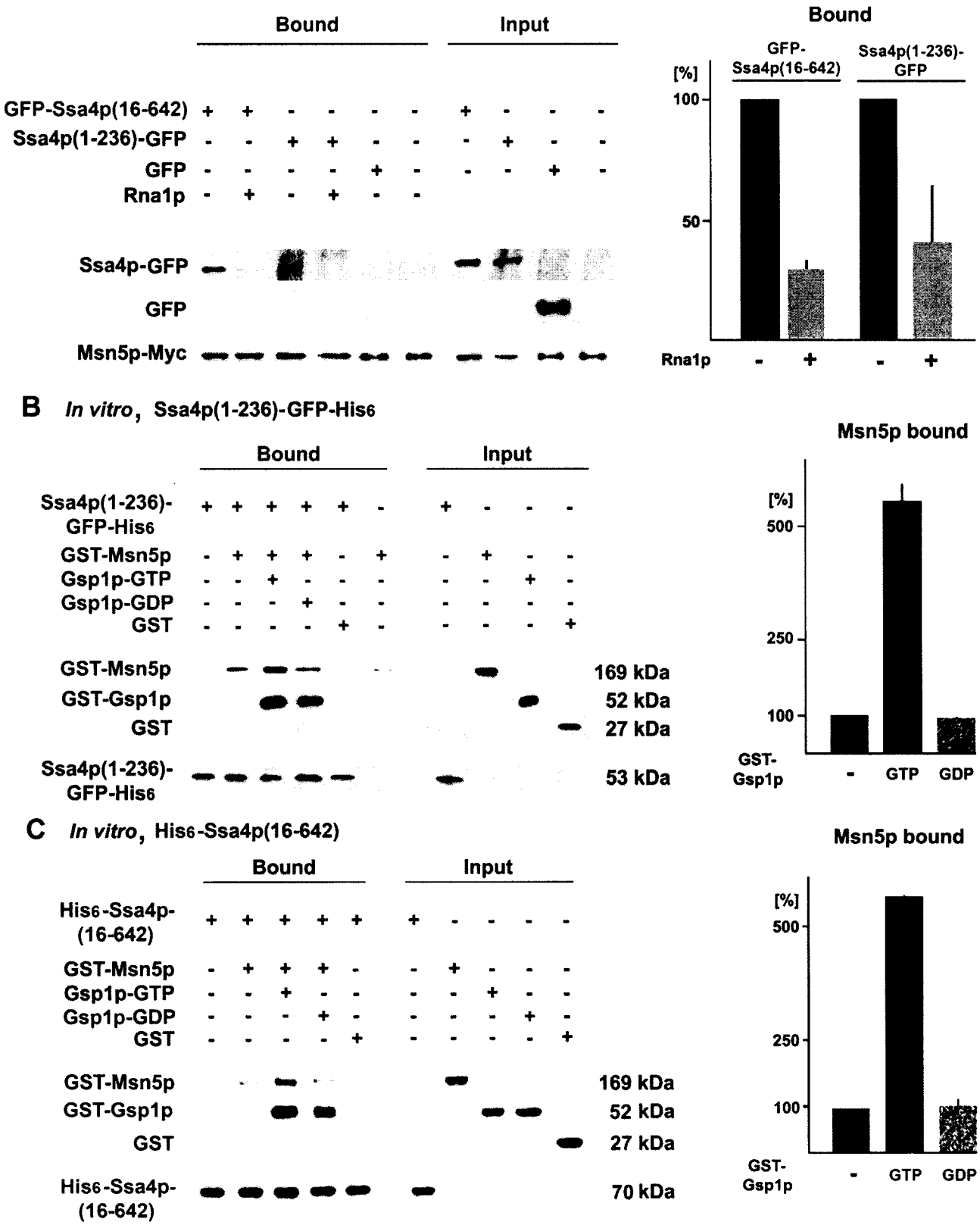

Fig 3.3 Ssa4p(1-236)-GFP associates with Myc-tagged Msn5p in growing cells and in vitro.

Fig 3.3 Ssa4p(1-236)-GFP associates with Myc-tagged Msn5p in growing cells and *in vitro*.

(A) Msn5p-containing protein complexes were purified from growing cells as described in Experimental procedures and analyzed by Western blotting with antibodies against GFP or the Myc-epitope. (B) Pull-down experiments were carried out with purified Ssa4p(1-236)-GFP-His6, GST-Msn5p, GST-Gsp1pQ71L (Gsp1p-GTP) or GST-Gsp1p (Gsp1p-GDP) as shown in the figure. GFP-His6 was used in control experiments. (C) The experiments shown in part B were carried out with His6-Ssa4p(16-642). The amounts of GFP-Ssa4p(16-642) and Ssa4p(1-236)-GFP bound to Msn5p-Myc (part A) or Myc-tagged Msn5p co-purifying with Ssa4p (parts B, C) were measured after Western blotting by densitometry of the film (Experimental procedures).

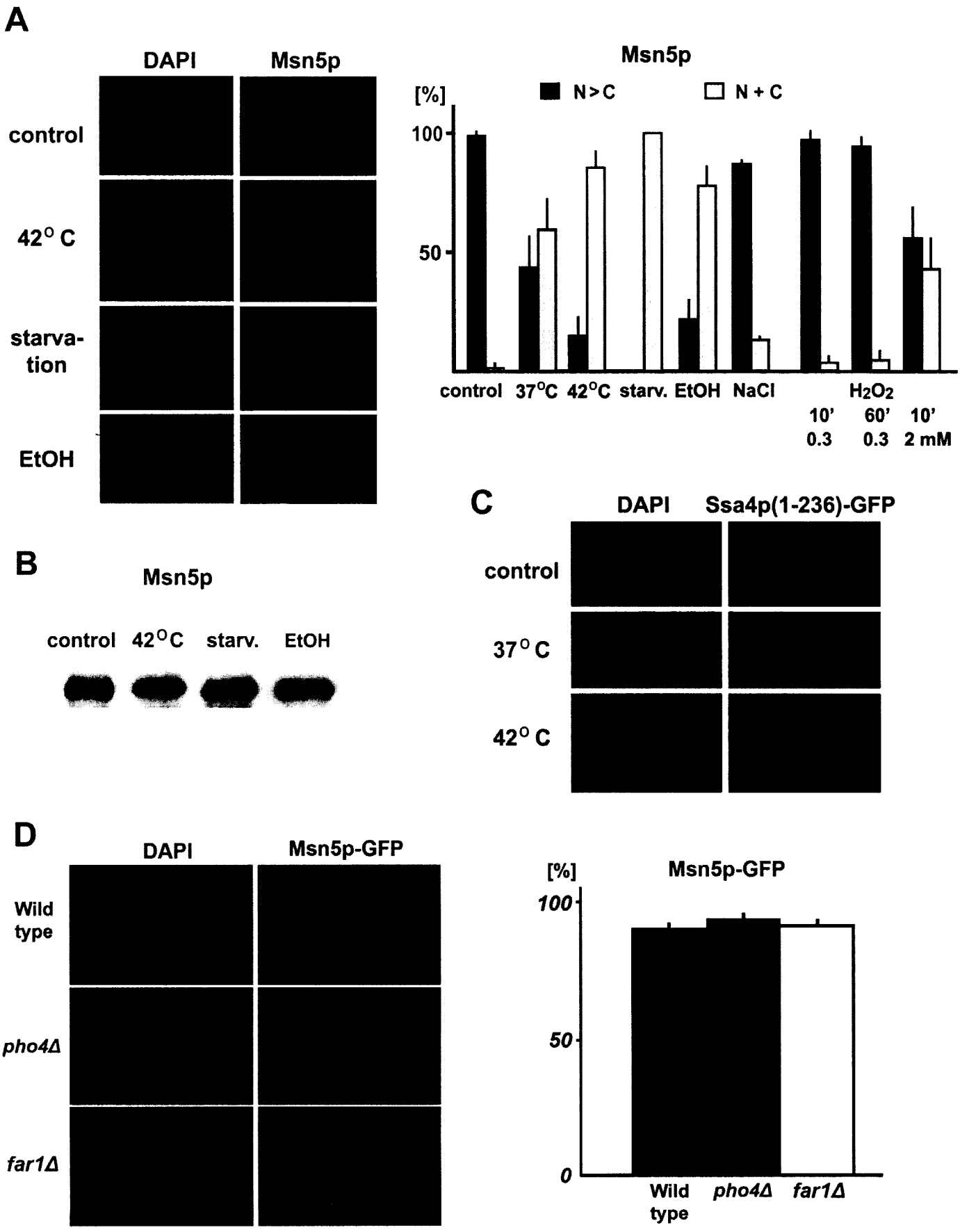

Fig 3.4 Localization of Msn5p is sensitive to several forms of stress.

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Myc-tagged Msn5p was localized in yeast cells exposed to heat (6 h at 37°C, 1 h at 42°C), starvation, ethanol (10 min, 10%), NaCl (10 min, 0.4 M) or H_2O_2 (10 min, 0.3 mM; 60 min, 0.3 mM; 10 min, 2 mM). **A**. Myc-Msn5p was detected by indirect immunofluorescence and results were quantified as in Fig 3.1.**B**. Protein extracts were prepared for controls or cells exposed to heat (1 h at 42°C), starvation or ethanol (10 min, 10%). Comparable amounts of protein were probed with antibodies against the Myc-epitope. **C**. Ssa4p(1-236)-GFP accumulates in nuclei of heat-stressed cells. Ssa4p(1-236)-GFP was localized in controls and cells incubated for 6 h at 37°C or 1 h at 42°C. **D**. Msn5p-GFP was localized in wild-type cells or strains *pho4Δ* and *far1Δ*. Results are shown for three separate experiments, for each at least 100 cells were scored.

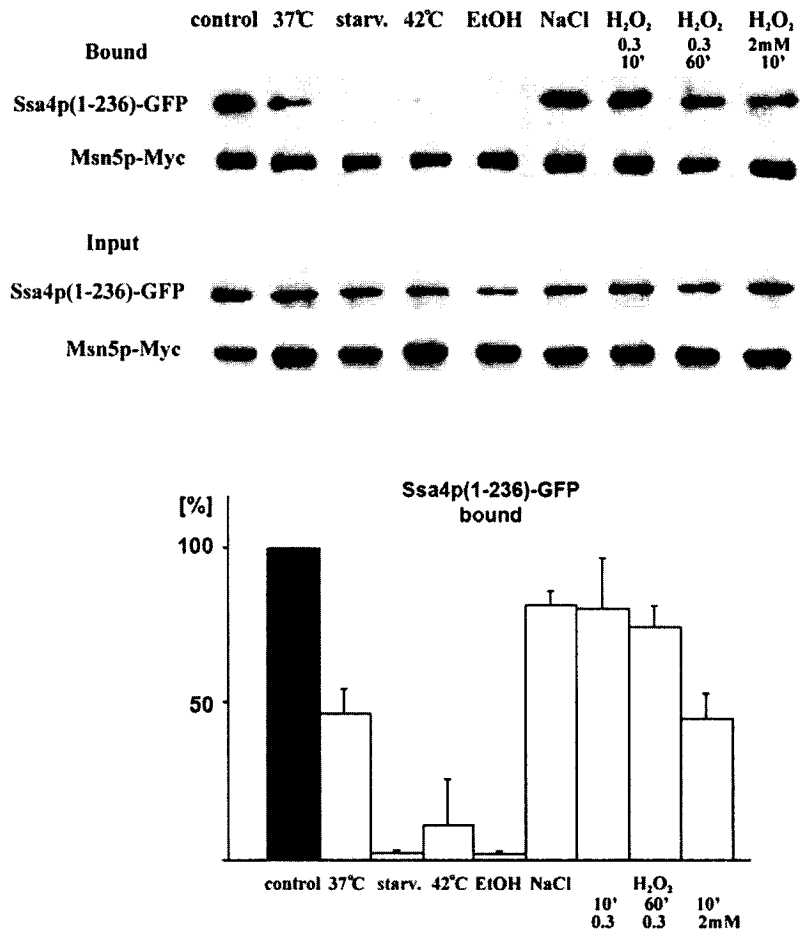

Fig 3.5 Stress regulates the interaction between Msn5p and Ssa4p(1-236)-GFP.

Fig 3.5 Stress regulates the interaction between Msn5p and Ssa4p(1-236)-GFP.

Msn5p-containing complexes were isolated from growing cells and binding was quantified as in Fig 3.3A. The amount of Ssa4p(1-236)-GFP bound under control conditions was defined as 1. Means and SD are shown for at least three independent experiments for each condition.

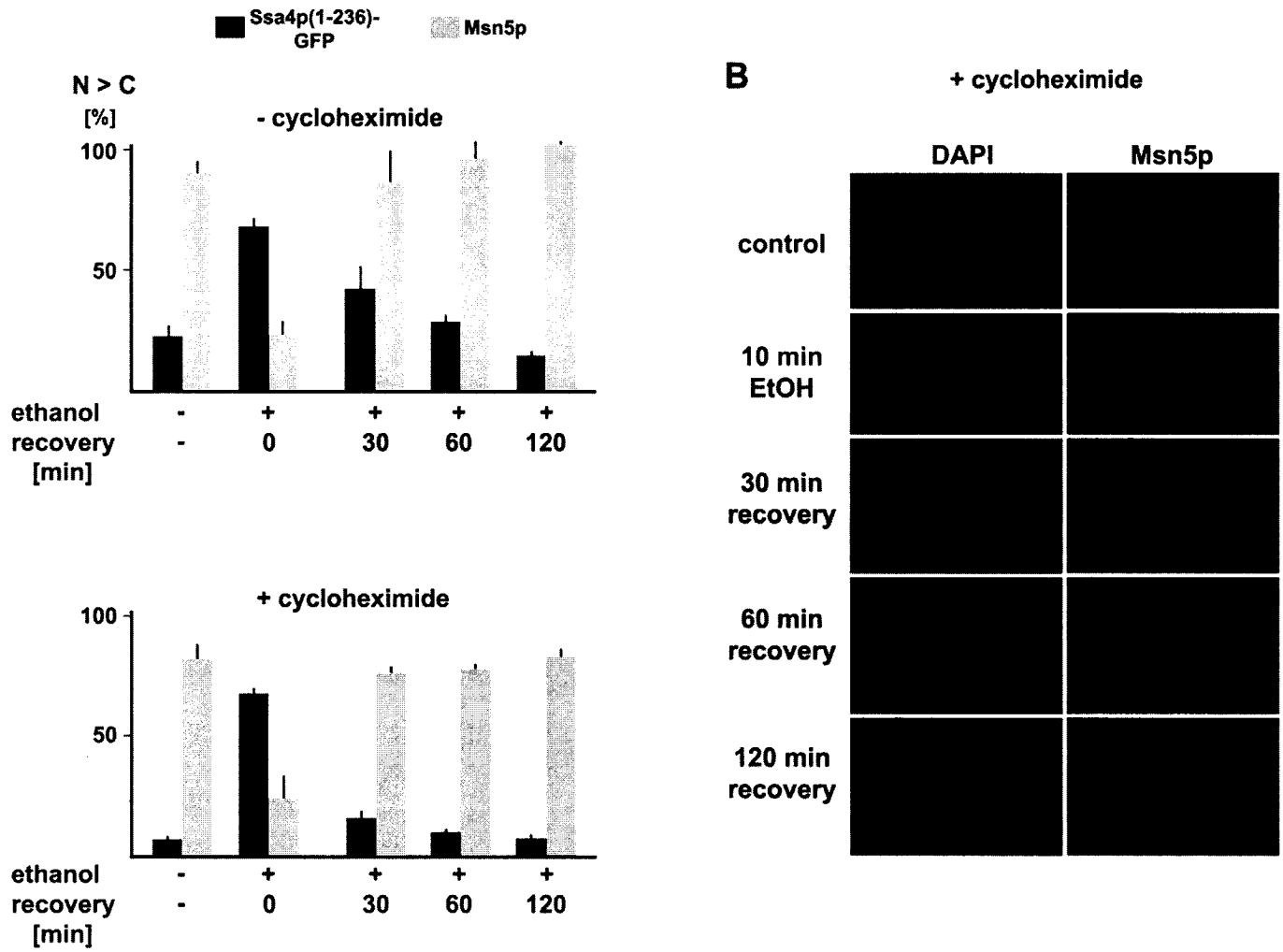

Fig 3.6 Msn5p localizes to the cytoplasm upon ethanol treatment and relocates to the nucleus during recovery.

(A) Yeast cells synthesizing Ssa4p(1-236)-GFP and Myc-tagged Msn5p were kept under non-stress conditions (- ethanol, - recovery) or treated for 10 min with 10% ethanol. Cells recovered in ethanol-free medium for the times indicated. Ssa4p(1-236)-GFP and Msn5p-Myc were located for the different time points by fluorescence microscopy or indirect immunofluorescence. The localization of Ssa4p(1-236)-GFP and Msn5p-Myc was quantified as described for Fig 3.1. In the lower panel cycloheximide was present at 100 µg/ml throughout the stress and recovery period. (B) The localization of Msn5p-Myc was monitored in control, ethanol-stressed and recovering cells by indirect immunofluorescence with antibodies against the Myc-epitope.

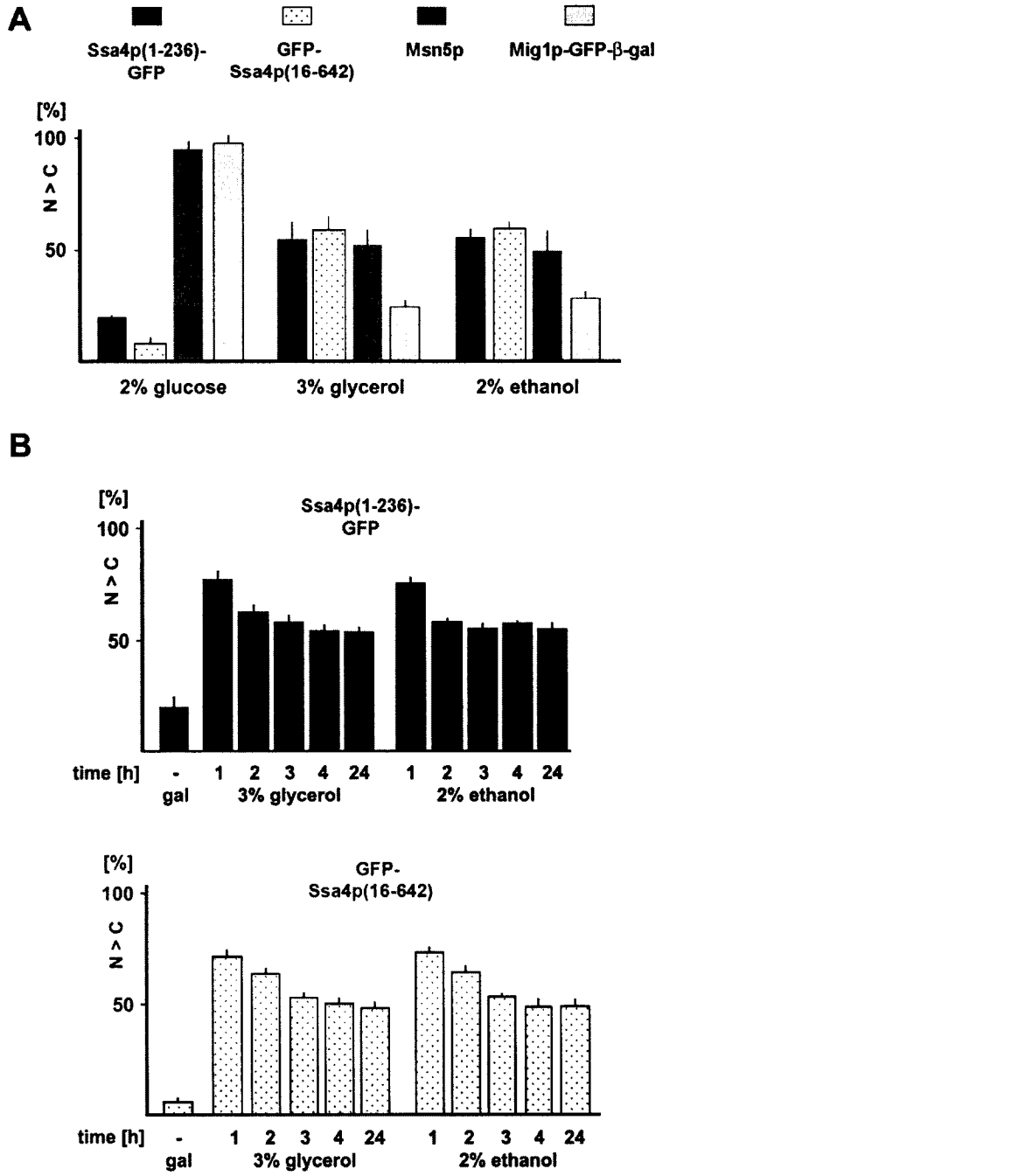

Fig 3.7 Non-fermentable carbon sources alter the distribution of Ssa4p(1-236)-GFP, GFP-Ssa4p(16-642) and Msn5p.

(A) Yeast cells synthesizing Ssa4p(1-236)-GFP, GFP-Ssa4p(16-642), Myc-tagged Msn5p or Mig1p(261-400)-GFP-β-galactosidase [Mig1p-GFP-β-gal] were grown overnight in minimal medium supplemented with 2% glucose, 3% glycerol or 2% ethanol. Msn5p and GFP-containing reporter proteins were localized in three independent experiments; at least 100 cells were scored in each experiment for the distribution of carrier and cargo. (B) Yeast cells were grown overnight on minimal medium with 2% galactose to induce the synthesis of Ssa4p(1-236)-GFP or GFP-Ssa4p(16-642), collected by centrifugation, washed and resuspended in minimal medium containing 3% glycerol or 2% ethanol. Samples were fixed 1, 2, 3, 4 or 24 h after change of the carbon source and the distribution of Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) was measured as described for part (A).

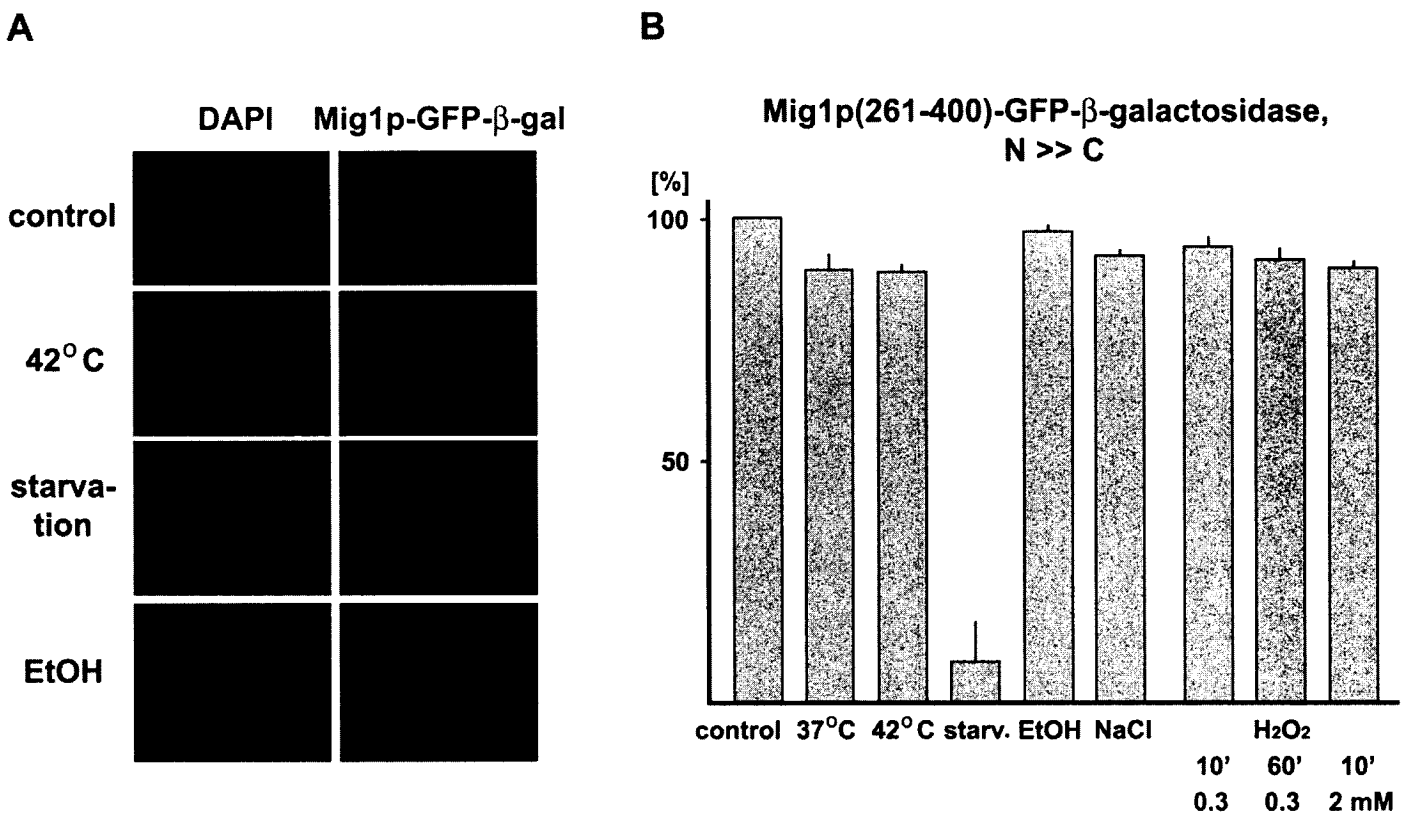

Fig 3.8 Mig1p relocates to the cytoplasm of starving cells, but not in response to other stresses.

Yeast cells synthesizing Mig1p(261-400)-GFP-β-galactosidase (Mig1p-GFP-β-gal) were incubated for 6 h at 37°C, 1 h at 42°C, starved, treated with 10% ethanol (10 min), 0.3 mM H2O2 (10 min, 60 min) or 2 mM H2O2 (10 min). Nuclear accumulation of the reporter protein Mig1p-GFP-β-gal was monitored for at least 100 cells in each of three independent experiments. Means and standard deviations are shown. (A) Mig1p(261-400)-GFP-β-galactosidase was located by fluorescence microscopy and results were quantified after exposure to different stressors (B).

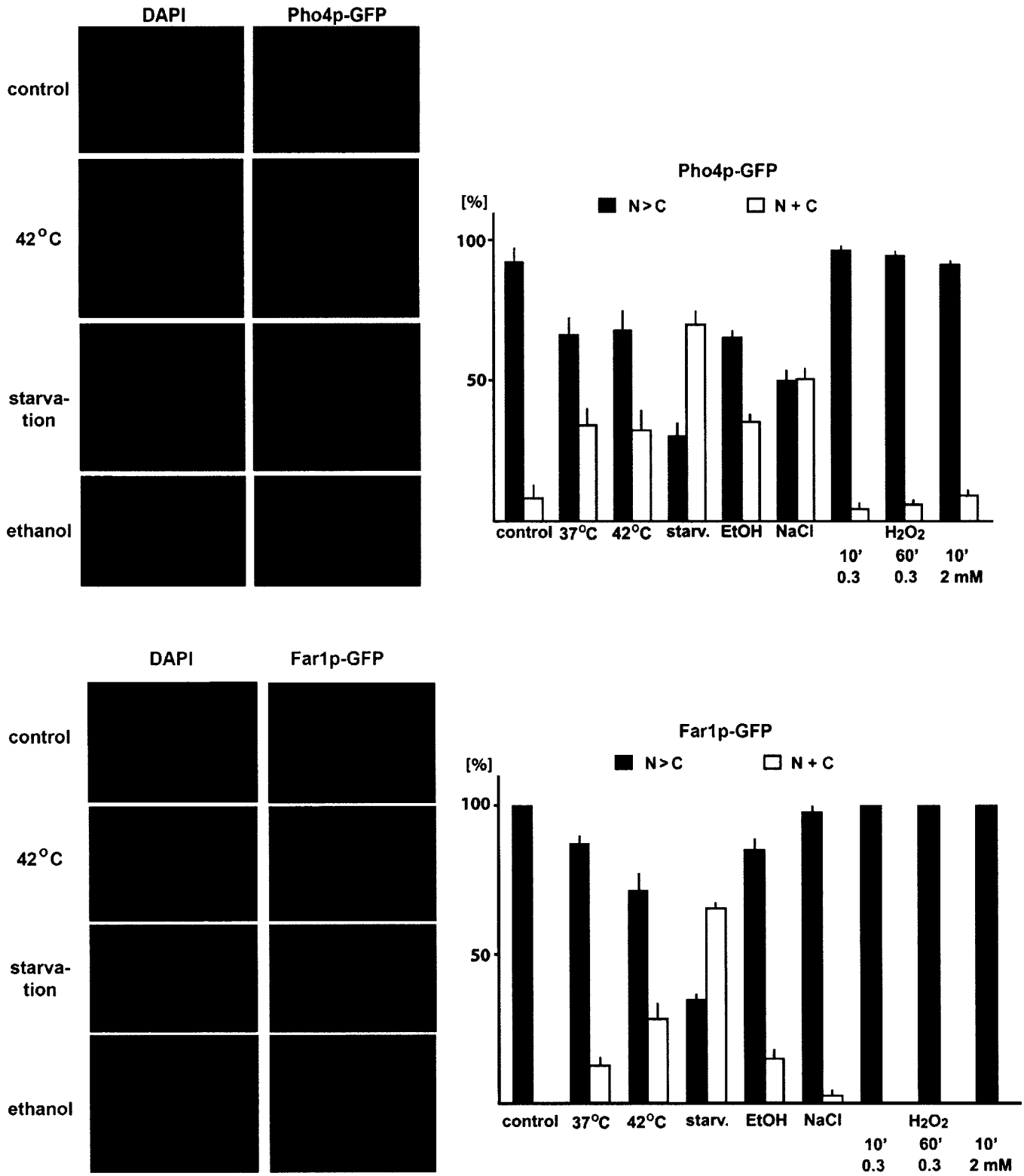

Fig 3.9 The localization of Pho4p and Far1p is sensitive to stress.

The distribution of Pho4p-GFP and Far1p-GFP was analyzed under the same conditions as shown for Fig 3.8.

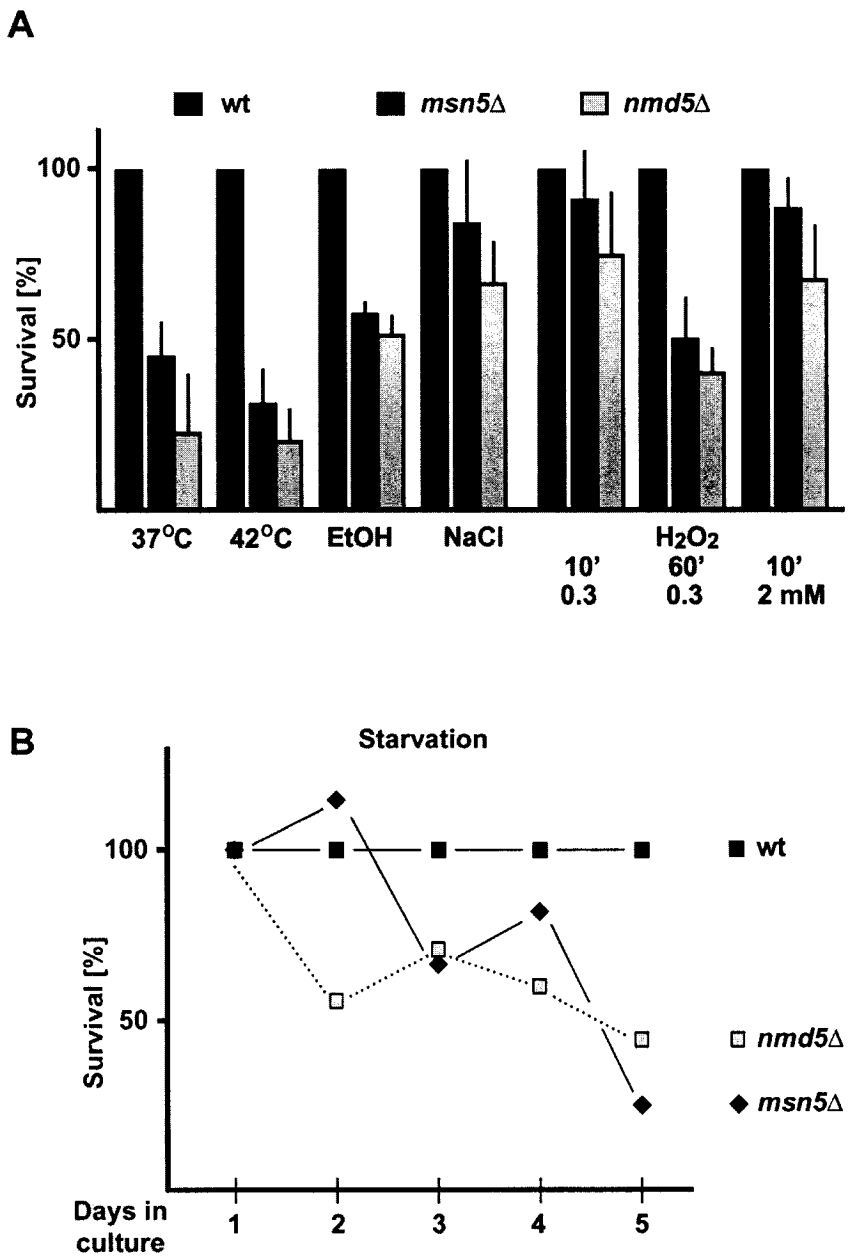

Fig 3.10 Mutant strains *msn5*Δ and *nmd5*Δ show increased sensitivity to stress.

Wild type (W303) and mutant strains lacking a functional *MSN5* or *NMD5* gene were stressed as detailed in Fig3.4 and immediately plated out. The colony formation of mutants and wild type cells was compared. Survival of wild type cells was defined as 100% for each of the different stresses. Under all stress conditions tested *msn5*Δ and *nmd5*Δ displayed a lower viability than the wild type strain. Means and S.D. for three independent experiments are shown.

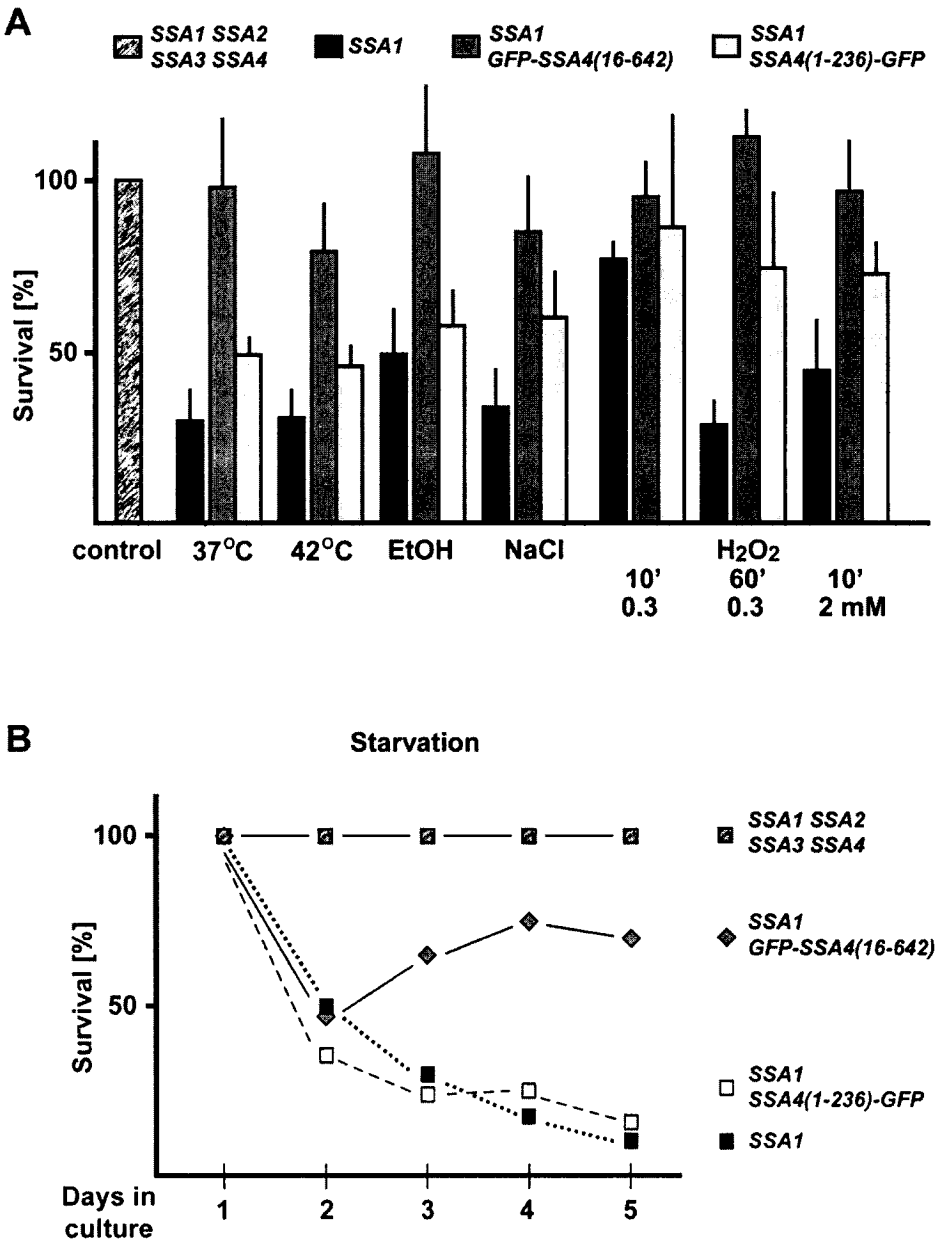

Fig 3.11 Ssa4p increases the viability of stressed yeast cells.

Yeast cells carrying genes *SSA1 SSA2 SSA3 SSA4* (control), *SSA1*, *SSA1* and *GFP-SSA4(16-642)* or *SSA1* and *SSA4(1-236)-GFP* were incubated under the conditions given in Fig3.4 and plated out immediately. Survival rates for *SSA1 SSA2 SSA3 SSA4* were defined as 100%. Means and S.D. are depicted for three independent experiments. For all treatments *GFP-SSA4(16-642)* significantly increased the viability of stressed *SSA1* cells ($p < 0.02$, student's t-test).

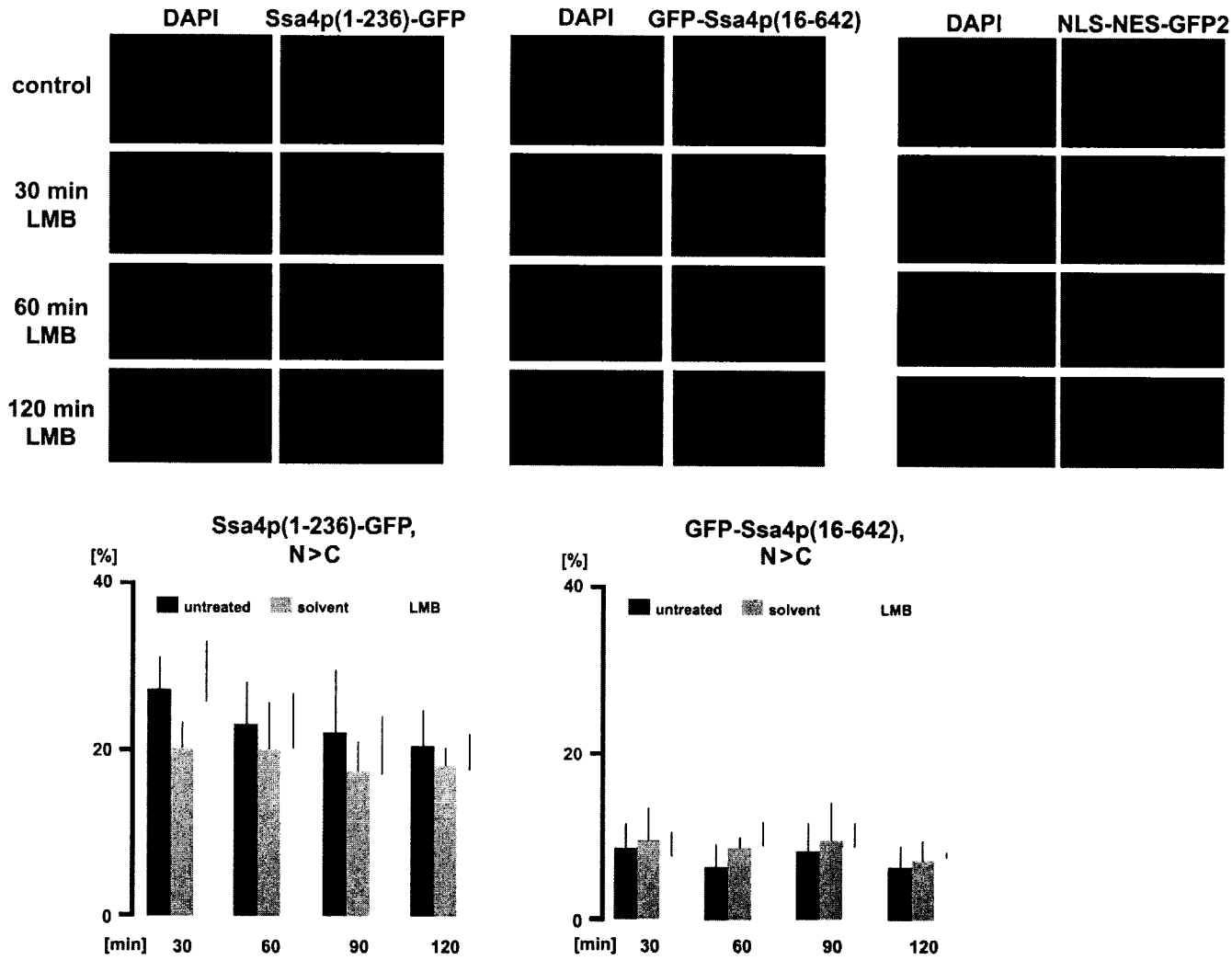

Fig 3.12 Nuclear export of Ssa4p(1-236)-GFP or GFP-Ssa4p(16-642) does not require the carrier Xpo1p.

Yeast cells carrying the LMB-sensitive CRM1 T539C allele were grown at room temperature and exposed to LMB for the times indicated. For comparison, the fluorescent shuttling protein NES-NLS-GFP2 was analyzed. NES-NLS-GFP2 accumulates rapidly in nuclei upon addition of LMB, whereas Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) fail to concentrate in nuclei under the same conditions. The location of Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) was quantified in untreated controls, cells incubated with the solvent only or treated with 100ng/ml LMB. The percentage of cells that accumulate Ssa4p(1-236)-GFP or GFP-Ssa4p(16-642) in nuclei (N>C) was determined for three independent experiments; at least 100 cells were scored for each experiment. Means and standard deviation are shown.

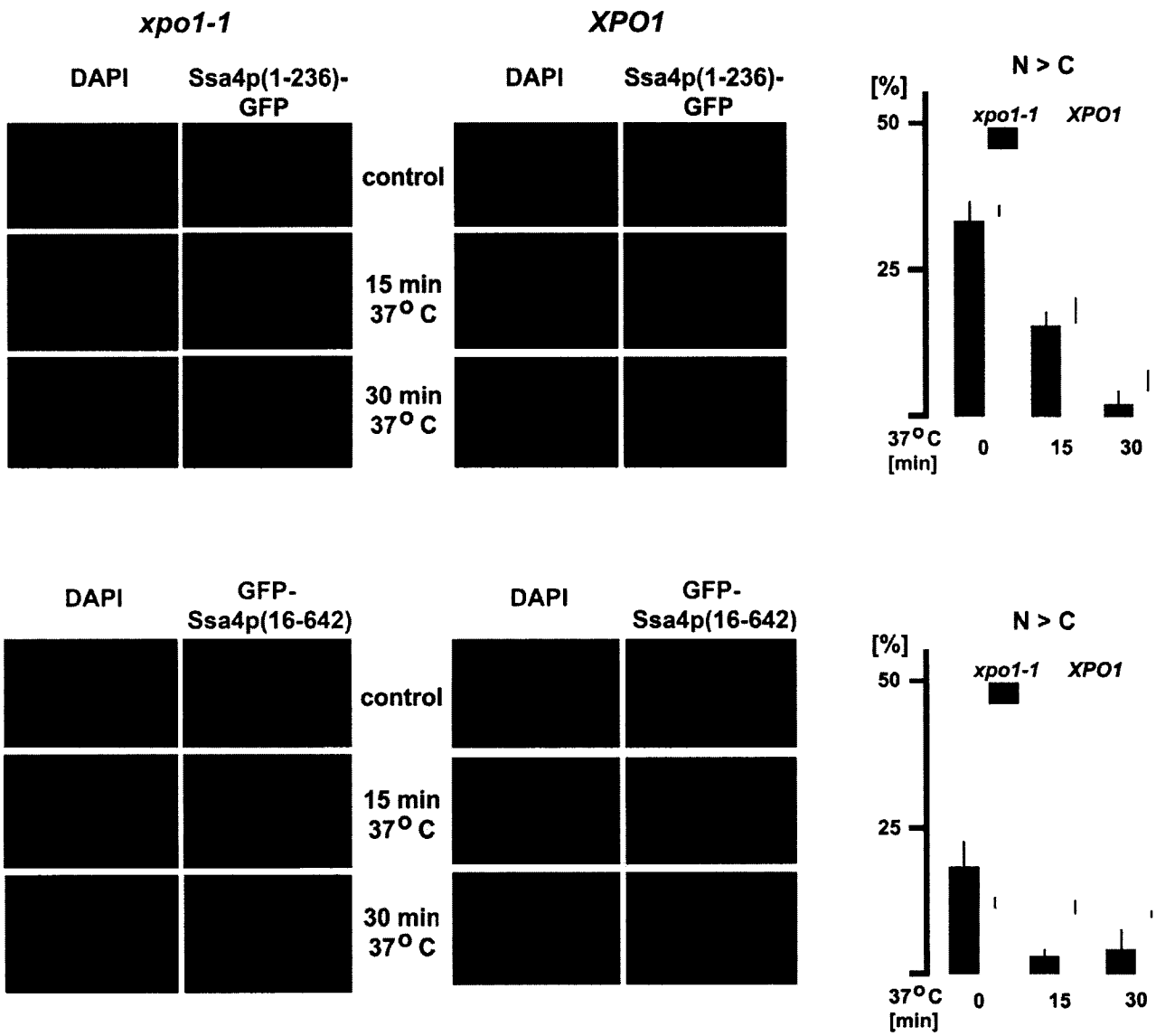

Fig 3.13 Xpo1p is not essential for Ssa4p nuclear export.

Mutant *xpo1-1* cells were incubated at room temperature or heat-treated for 15 min and 30 min at 37°C to inactivate the temperature sensitive carrier. Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) were localized by fluorescence microscopy. Quantification was carried out as described for Fig3.1.

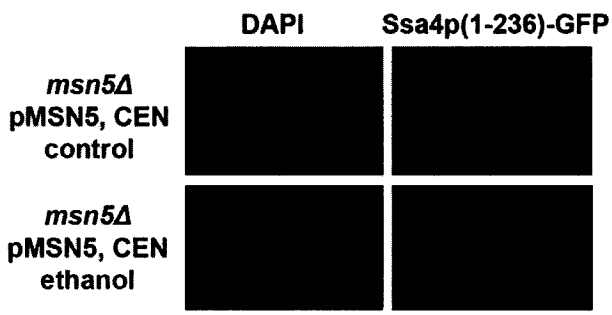

Fig 3.14 *MSN5* provided on a centromeric plasmid rescues ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP in *msn5*Δ cells.

Wild type *MSN5* was introduced on a centromeric plasmid and Ssa4p(1-236)-GFP was localized as in Fig 3.1..

CHAPTER 4

The localization of nuclear exporters of the importin-β family is regulated by Snf1 kinase, nutrient supply and stress

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4.1 Connecting text

As shown in chapter 3, Msn5p-mediated nuclear export of Ssa4p is important for the regulation of cargo Ssa4p distribution under normal conditions. It is the first report that not only Ssa4p, but also nuclear exporter Msn5p localization is regulated by stress as well as carbon sources composition. The results suggest that Msn5p subcellular localization could play a role in the regulation of specific nuclear transport process. There are four members of the nuclear transport receptor importin-β family that function as nuclear exporters in yeast *S. cerevisiae*. Here we further studied the effects of nutritional status and stress on regulation of the localization of these four exporters. Snf1 kinase was identified to control the distribution of all exporters. And the role of MAPK pathway was detected in exporter localization. The results imply that the localization of nuclear exporters is regulated by multiple mechanisms including the physiological condition of cells and the environmental stress. This manuscript is submitted and in the review as ***Quan X;*** *Yu J; Bussey H, Stochaj U. The localization of nuclear exporters of the importin-beta family is regulated by Snf1 kinase, nutrient supply and stress. BBA - Molecular Cell Research (Submit Date: Nov 23, 2006, Manuscript No.: BBAMCR-06-380).*

4.2 Abstract

In the budding yeast S. cerevisiae, four members of the importin-β family of nuclear carriers, Xpo1p, Cse1p, Msn5p and Los1p, function as exporters of protein and tRNA. Under normal growth conditions these exporters are predominantly associated with nuclei. We demonstrate that Snf1 kinase, a key regulator of cell growth and a metabolic sensor, controls the distribution of all exporters. Moreover, a switch from fermentable to non-fermentable carbon sources relocates the carriers, suggesting a link to the nutritional status of the cell. In addition, stress regulates the proper localization of exporters, and mislocalization was observed upon exposure to heat, ethanol and starvation. Stress may activate the MAPK cell integrity cascade, and we tested the role of this pathway in exporter localization. Under non-stress conditions, the proper localization of Cse1p and Xpo1p requires kinases of the cell integrity cascade. By contrast, Msn5p and Los1p rely on the MAPK module to relocate to the cytoplasm when cells are stressed with ethanol. Our results indicate that the association of nuclear exporters with nuclei is controlled by multiple mechanisms that are organized in a hierarchical fashion and linked to the physiological state of the cell.

4.3 Introduction

Transport of molecules between cytoplasmic and nuclear compartments is a fundamental biological process conserved amongst eukaryotes. Nuclear

trafficking is required for proper growth, development, and the adaptation to environmental changes, all of which depend on nuclear export of transcriptional regulators and other components [Hood, 1999; Poon, 2006]. Trafficking between nucleus and cytoplasm is mediated by specialized carriers that translocate proteins and RNA across the nuclear envelope [Ström, 2001; Mosammaparast, 2004; Weis, 2003; Harel, 2004]. The budding yeast S. cerevisiae has 14 members of the importin- β, or karyopherin-β, family which are classified into importins and exportins, depending on whether they move cargo in or out of the nucleus. Four members of the importin-β family function as nuclear exporters; Xpo1p/Crm1p, Cse1p, Msn5p and Los1p transport protein or tRNA across nuclear pore complexes (NPCs). In the presence of Ran/Gsp1p-GTP in the nucleus, these carriers generate trimeric export complexes consisting of Gsp1p-GTP/carrier and their respective cargo [Ström, 2001; Mosammaparast, 2004; Weis, 2003; Harel, 2004]. Once in the cytoplasm, GTP hydrolysis dissociates export complexes, thereby liberating cargo, and the carrier which can return to the nucleus for another round of export. Following this mechanism, the exporter Xpo1p/Crm1p moves proteins or ribosomal subunits from nucleus to cytoplasm by recognizing hydrophobic leucine-rich nuclear export signals, whereas Cse1p is specialized for export of the adapter protein Srp1p/importin-α [Stade, 1997; Johnson, 2002; Solsbacher, 1998; Hood, 1998]. Msn5p is different from other importin-β like carriers as it can function both in nuclear import and export. Among the cargoes for Msn5p are several transcription factors that regulate nutrient-dependent gene expression, like Mig1p and Pho4p, the cell cycle regulator Swi6p and the hsp70, Ssa4p [DeVit, 1999; Kaffman, 1998; Queralt, 2003; Quan, 2006]. The importin-β

family member Los1p promotes nuclear export of tRNAs [Sarkar, 1998; Steiner-Mosonyi, 2004].

We previously identified Msn5p as the nuclear export carrier Ssa4p in budding yeast and demonstrated that Msn5p localization is controlled by stress and carbon source [Quan, 2006]. It is not known whether other exporters share these properties. The presence of nutrients, including the carbon source, regulates protein synthesis, gene expression and the activities of many proteins. Snf1 kinase, a highly conserved eukaryotic serine/threonine protein kinase, is an essential regulator of these processes [Sanz, 2003; Schüller, 2003; Hardie, 1998]. Snf1 kinase not only controls cell growth and transcription, but also the response to nutrient limitation, heat and osmotic stress [Sanz, 2003]. Like Snf1, the TOR (target of rapamycin) kinase pathway is at the center of metabolic and growth control in response to nutrient availability [Heitman, 1991; Wullschleger, 2006; Reiling, 2006; Virgilio, 2006]. TOR complex 1 can be inhibited with the antifungal antibiotic rapamycin, which interferes with growth in yeast and higher eukaryotes [Virgilio, 2006]. Moreover, the TOR and Snf1 kinase pathways converge to respond to nitrogen or carbon source availability [Mayordomo, 2002; Bertram, 2002].

One of the key regulators that is downstream of TOR function and also essential for the response to many forms of stress is the Pkc1p-cell integrity pathway [Torres, 2002; Gustin, 1998; Heinisch, 1999; Levin, 2005]. Following rapamycin treatment or plasma membrane stress, sensors in the plasma membrane, including Wsc1p and Mid2p, promote the activation of Pkc1p and thereby of the downstream cell integrity MAPK cascade. This MAPK module

consists of the four kinases Bck1p (MAPKKK), Mkk1p/Mkk2p (MAPKK) and Mpk1p (MAPK), which can interact with phosphatases or other kinases [reviewed in Gustin, 1998; Heinisch, 1999; Levin, 2005]. Although Pkc1p is the most prominent activator of this MAPK cascade, input from other pathways may regulate this signaling route [Gustin, 1998; Heinisch, 1999; Levin, 2005; Deng, 2005].

Snf1 kinase, the cell integrity pathway and nuclear transport are essential components necessary to respond to nutrient availability or stress. We explored here a possible role of these protein kinases as well as nutrient supply in regulating nuclear exporters of the importin-β family.

4.4 Materials and methods

4.4.1 Strains, plasmids and growth conditions

Wild type and mutant yeast strains used in this study are shown in Table 4.1. Plasmids encoding GFP-tagged Xpo1p, Cse1p, Msn5p or Los1p were kindly provided by K. Weis, G. Schlenstedt, L. Huang, and D. Mangroo. *SNF1* wild type and mutant cells were a gift of M. Johnston [Lutfiyya, 1998]. Cells were grown in drop out medium containing 2% glucose, 2% galactose, 3% glycerol or 2% ethanol as carbon source. Yeast cells were transformed using standard procedures; transformants were selected and grown on complete minimal medium lacking the amino acids or nutrients used for selection. The growth media of *pkc1Δ* and yeast strains mutated in components of the cell integrity pathways were supplemented with 10% sorbitol.

4.4.2 Localization of GFP-fusion proteins

Fusion proteins containing the GFP-tag were localized as described [Quan, 2006]. In brief, samples were analyzed with a Nikon Optiphot microscope or Zeiss LSM 510 at 1,000 X magnification. The localization of proteins was monitored by visual inspection of the specimens. To avoid the non-linear range of fluorescence signals, cells highly overexpressing one of the GFP-tagged carriers were excluded from further analyses. A minimum of 100 cells were scored in each experiment, and at least three independent experiments were performed for each condition. N>C denotes nuclear accumulation with weak signals detected in the cytoplasm; NE, accumulation at the nuclear envelope; N≥C, weak nuclear accumulation; N+C, comparable fluorescence signal in nucleus and cytoplasm. For each experiment, identical settings were used for imaging. Negatives and confocal images were processed with Adobe Photoshop 8.0.

4.4.3 Growth on different carbon sources

To monitor the effect of carbon sources, cells were cultured overnight in selective media with 2% galactose, collected by centrifugation and resuspended in fresh medium containing 2% glucose, 2% ethanol or 3% glycerol. After transfer to fresh medium, cells were grown for 4 hours, fixed and further treated as described [Quan, 2006].

4.4.4 Rapamycin treatment

Yeast cells synthesizing one of the GFP-tagged exporters were grown overnight in selective medium with 2% galactose. Rapamycin was added from freshly prepared stocks (50 μM) to a final concentration of 500 nM. Controls were incubated in the absence of rapamycin. After 3-hour incubation cells were fixed and processed for microscopy [Quan, 2006].

4.4.5 Stress assays

Yeast cells were cultured overnight at room temperature in selective medium containing 2 % galactose and exposed to different forms of stress; heat: 6 hours 37°C or 1 hour 42°C; ethanol: 10 min 10% (final concentration); osmotic stress: 10 min 0.4 M NaCl (final concentration); oxidative stress: 10 min or 1 hour 0.3 mM H_2O_2 (mild), and 10 min 2 mM H_2O_2 (severe). Cells were starved by continuous growth for 4 days in the same medium.

4.4.6 Western blot analysis

Western blotting with antibodies against GFP was carried out as described previously [Quan, 2006].

4.5 Results

4.5.1 The localization of nuclear exporters is controlled by Snf1 kinase, by carbon source, and is sensitive to rapamycin

To identify a possible link between the nutritional state of the cell and the regulation of nuclear exporters, we analyzed yeast cells that carry a knockout of the *SNF1* gene. In mutant as well as in isogenic wild type cells, GFP-tagged

exporters were localized by fluorescence microscopy. For wild type cells under non-stress conditions all of the exporters were predominantly associated with nuclei (Fig 4.1A). However, their distribution was significantly altered in *snf1Δ* cells. For instance, Xpo1p-GFP nuclear accumulation decreased from 91% (wild type) to about 47% in the mutant strain, with relocation to the cytoplasm. Similar results were obtained for Los1p-GFP, GFP-Cse1p and Msn5p-GFP (Fig 4.1A).

Since Snf1 kinase is crucial for the regulation of glucose-sensitive gene expression, we further investigated the effects of different carbon sources on nuclear exporters. In the presence of glucose all of the carriers were mostly nuclear, whereas incubation with glycerol or ethanol induced a substantial relocation to the cytoplasm (Fig 4.1B). As reported previously, the GFP-tag was not affected by the carbon source and both nuclear as well as cytoplasmic under all conditions [Quan, 2006].

Rapamycin treatment mimics amino acid starvation, thereby signaling stress and causing growth inhibition [Heitman, 1991; Wullschleger, 2006; Reiling, 2006]. As shown in Fig 4.2 incubation with rapamycin induced a significant mislocalization of all nuclear exporters; albeit to a lesser extent than glycerol and ethanol.

Taken together, our results suggest that the nucleocytoplasmic distribution of export carriers is controlled by Snf1 kinase, the carbon source and TOR.

4.5.2 Stress relocates nuclear exporters

Results described above show the importance of nutrients for the proper distribution of nuclear exporters. Thus, we further investigated the effect of starvation and other forms of stress.

Mostly nuclear in unstressed wild type cells, (Fig 4.1A and 4.3), elevated levels of all carriers were present in the cytoplasm upon exposure to certain stressors. This redistribution of carriers can not simply be attributed to stress-induced degradation, since similar amounts of transporters were detected in crude extracts of control and stressed cells (Fig 4.3A-C, and data not shown).

As depicted in Fig 4.3, individual carriers differ in sensitivity towards a particular form of stress. For instance, when exposed to heat shock or ethanol, substantial levels of all exporters were detected in the cytoplasm (Fig 4.3A-C and Quan, 2006). Starvation had a strong effect on Cse1p and Msn5p (Fig 4.3B and Quan, 2006), but was less efficient in relocating Xpo1p and Los1p (Fig 4.3A, C). The distribution of all carriers was somewhat altered by osmotic and oxidative stress, with NaCl having its greatest effect on Xpo1p (Fig 4.3).

Taken together, for all yeast importin-β like exporters the nuclear versus cytoplasmic localization can be modulated by stress. However, individual exporters display distinct patterns of sensitivity towards specific types of stress.

4.5.3 The nuclear association of Xpo1p and Cse1p, but not Los1p and Msn5p, depends on kinases of the cell integrity pathway under normal growth conditions

Some forms of stress, in particular heat, lead to the activation of Pkc1p and the cell integrity MAPK pathway. However, these signaling pathways may also regulate nuclear exporter function under non-stress conditions. To address this question, we located nuclear exporters in strains mutant in components of the Pkc1p-cell integrity pathway. Surprisingly, in the absence of stress the proper distribution of Xpo1p-GFP relied on mutants *bck1Δ, mkk1Δ, mkk2Δ* and *mpk1Δ.* Similarly, Cse1p required kinases of the MAPK module, Rom1p, (GDP/GTP exchanger of the small GTPase Rho1p), the plasma membrane sensor Mid2p, and Pmt2p an O-mannosyl transferase that modifies Mid2p. None of the exporters required Pkc1p for their association with nuclei (Fig 4.4B) and none of the mutant strains mislocalized Msn5p-GFP or Los1p-GFP in unstressed cells (Fig 4.4A, B).

4.5.4 Ethanol-induced mislocalization of Msn5p and Los1p requires protein kinases of the cell integrity MAPK module

Ethanol stress may alter the fluidity of the plasma membrane and thereby activate the cell integrity kinase cascade. We therefore determined whether the ethanol-induced relocation of transporters to the cytoplasm (Fig 4.3) is altered in mutants that can not signal through this pathway. The release of Msn5p and Los1p from nuclei was drastically reduced in mutants *bck1Δ, mkk1Δ, mkk2Δ* and *mpk1Δ*, but not in *pkc1Δ* cells (Fig 4.5A, B). By contrast, Xpo1p and Cse1p efficiently redistributed to the cytoplasm in all of the kinase mutants (Fig 4.5B),

suggesting that their release from the nuclei of ethanol-stressed cells is regulated differently.

4.6 Discussion

We have analyzed the steady-state distribution of nuclear exporters of the importin-β family and demonstrate that they are subject to complex regulation on different levels. Our studies identify new links that connect the metabolic sensor Snf1 kinase and nutrient supply to the proper localization of exporter carriers. These links provide general regulatory components that contribute to the distribution of all exporters. A common denominator of this control mechanism could be the cell's metabolic status [Sanz, 2003; Schüller, 2003; Hardie, 1998; Heitman, 1991; Wullschleger, 2006; Reiling, 2006; Virgilio, 2006; Mayordomo, 2002; Bertram, 2002; Torres, 2002], which is likely to impinge on energy-consuming processes like nuclear export. Relocation of nuclear exporters to the cytoplasm may then reduce export efficiency by making only a portion of the carriers available in the nucleus.

On a different level, signaling through the MAPK cell integrity pathway [Gustin, 1998; Heinisch, 1999] regulates different classes of carriers. We defined Xpo1p and Cse1p as well as Msn5p and Los1p, respectively, as two groups that are similarly affected by this kinase module. For Xpo1p and Cse1p the kinase cascade is required for nuclear association under non-stress conditions, whereas Msn5p and Los1p rely on this pathway for their redistribution upon stress.

Although Pkc1p is the most important upstream regulator of the MAPK cell integrity cascade, our results suggest that input from Pkc1p is not essential to localize nuclear exporters. This could suggest that other upstream regulator(s) are involved in these signaling events. An example of Pkc1p-independent activation of the cell integrity pathway was recently reported in *S. pombe* [Deng, 2005], and similar mechanism(s) may control the localization of exporters in *S. cerevisiae* as well. A possible model to explain the effect of the MAPK module would propose that factors interacting with either group of carriers are regulated by the cell integrity pathway. Candidate components for this control are cytoplasmic anchors for Xpo1p and Cse1p or nuclear anchors that prevent the stress-induced release of Msn5p and Los1p. Alternatively, the cell integrity cascade may control the function of nucleoporins that are necessary for either group of carriers to move in and out of the nucleus.

Furthermore, we demonstrate that environmental stresses alter the association of exporters with the nucleus. Although all exporters are sensitive to stress, they show unique sensitivities towards specific insults, suggesting that some stresses control carriers on an individual basis. Here a testable hypothesis is that individual regulation is achieved by reversible posttranslational modifications of a particular carrier.

Our results are consistent with the idea that exporter localization is controlled in a hierarchical fashion. This complex regulation may provide a platform to

adjust the efficiency of nuclear export according to the changes in cellular metabolism or environmental conditions.

4.7 Acknowledgements

We thank Drs. L.S. Huang, D. Mangroo, G. Schlenstedt, and K. Weis for generous gifts of yeast strains and plasmids. We are grateful to N. Matusiewicz for technical assistance. This work was supported by grants from CIHR and Heart and Stroke Foundation of Quebec to US. US is a chercheur national of FRSQ. XQ was supported by a fellowship from FRSQ.

Table 4.1 Yeast strains used in this study.

Strain	Genotype
W303	*MATa ade2 his3 leu2 trp1 ura3*
snf1Δ	*MATa lys2-801::BM1499 snf1Δ*
BY4741(wt1)	*MAT*a, *his3Δ1, leu2Δ, met15Δ, ura3Δ*
wsc1Δ	*wsc1Δ his3Δ1, leu2Δ, met15Δ, ura3Δ*
rom1Δ	*rom1Δ his3Δ1, leu2Δ, met15Δ, ura3Δ*
mid2Δ	*mid2Δ his3Δ1, leu2Δ, met15Δ, ura3Δ*
pmt2Δ	*pmt2Δ his3Δ1, leu2Δ, met15Δ, ura3Δ*
BY4743(wt2)	*MATa/*a, *his3Δ1, leu2Δ, met15Δ, ura3Δ, lys2Δ*
mid2Δwsc1Δ	*mid2Δwsc1Δ, MATa/*a, *his3Δ1, leu2Δ, ura3Δ, lys2Δ*
pkc1Δ	*pkc1Δ, MATa/*a, *his3Δ1, leu2Δ, ura3Δ*
bck1Δ	*bck1Δ, MAT*a, *his3Δ1, leu2Δ, met15Δ, ura3Δ*
mkk1Δ	*mkk1Δ, MAT*a, *his3Δ1, leu2Δ, met15Δ, ura3Δ*
mkk2Δ	*mkk2Δ, MAT*a, *his3Δ1, leu2Δ, met15Δ, ura3Δ*
mpk1Δ	*mpk1Δ, MAT*a, *his3Δ1, leu2Δ, met15Δ, ura3Δ*

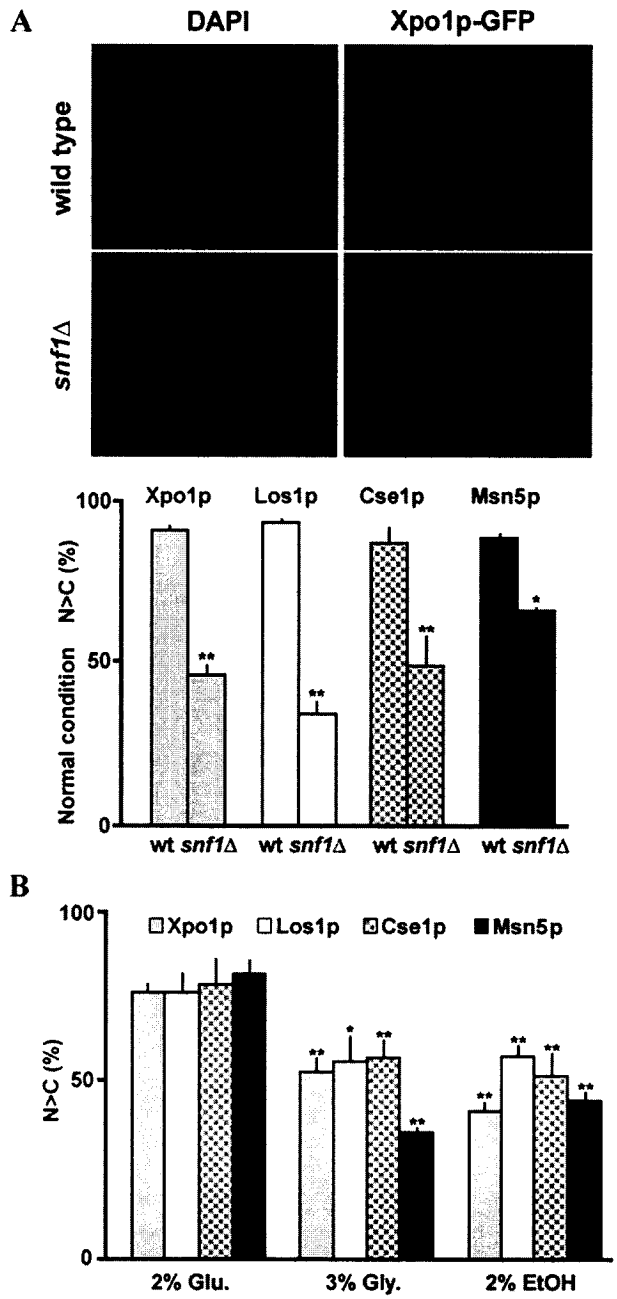

Fig 4.1 Snf1 kinase and the carbon source regulate nuclear exporters.

Fig 4.1 Snf1 kinase and nutrients regulate nuclear exporters.

(**A**) GFP-tagged export carriers were localized in wild type or isogenic mutant cells carrying a *snf1Δ* gene knockout. Fluorescent microscopy located Xpo1p-GFP; nuclei were stained with DAPI. The percentage of nuclear accumulation (N>C) was monitored for all export carriers in both wild type cells and *snf1Δ* cells. Standard deviation and Student's two-tailed t-test are shown, * $p<0.02$; ** $p<0.005$. (**B**) Export carriers are nuclear when cells grow on glucose and relocate to the cytoplasm in the presence of the non-fermentable carbon sources glycerol or ethanol. The percentage of nuclear accumulation (N>C) is shown as mean $\pm$ S.D. All data are based on at least three independent experiments.

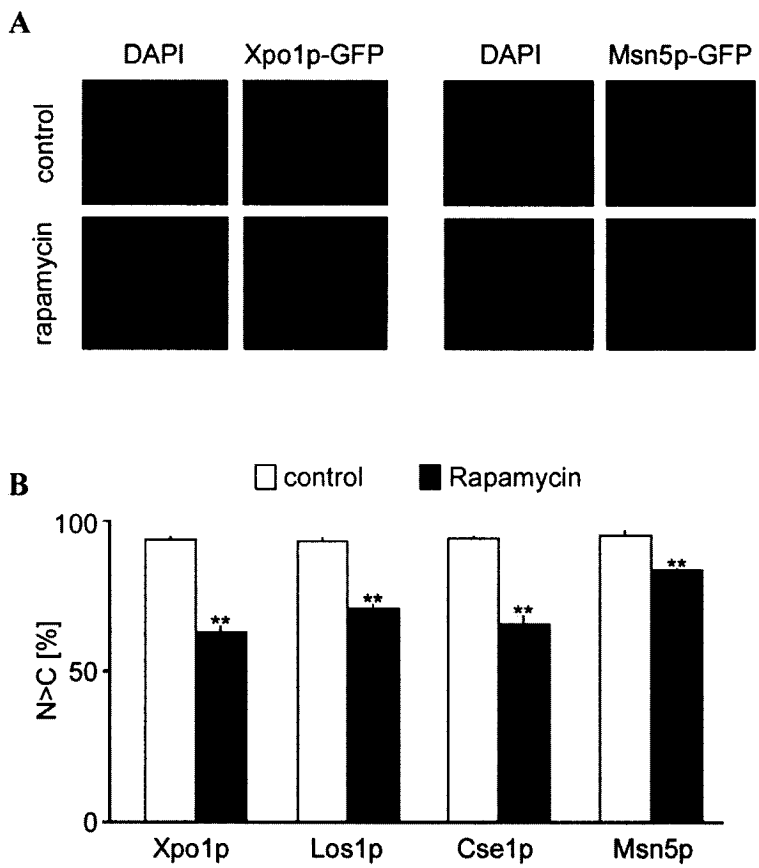

Fig 4.2 Rapamycin treatment changes the localization of all four nuclear exporters.

Fig 4.2 Rapamycin treatment changes the localization of all four nuclear exporters.

(**A**) Wild type cells synthesizing GFP-tagged carriers were incubated in the absence or presence of rapamycin. After a 3-hour incubation, cells were fixed and exporters localized by fluorescent microscopy. (**B**) Nuclear accumulation (N>C) was quantified in control and drug-treated cells. Means and standard deviations are shown for at least three independent experiments for each exporter, ** $p<0.001$.

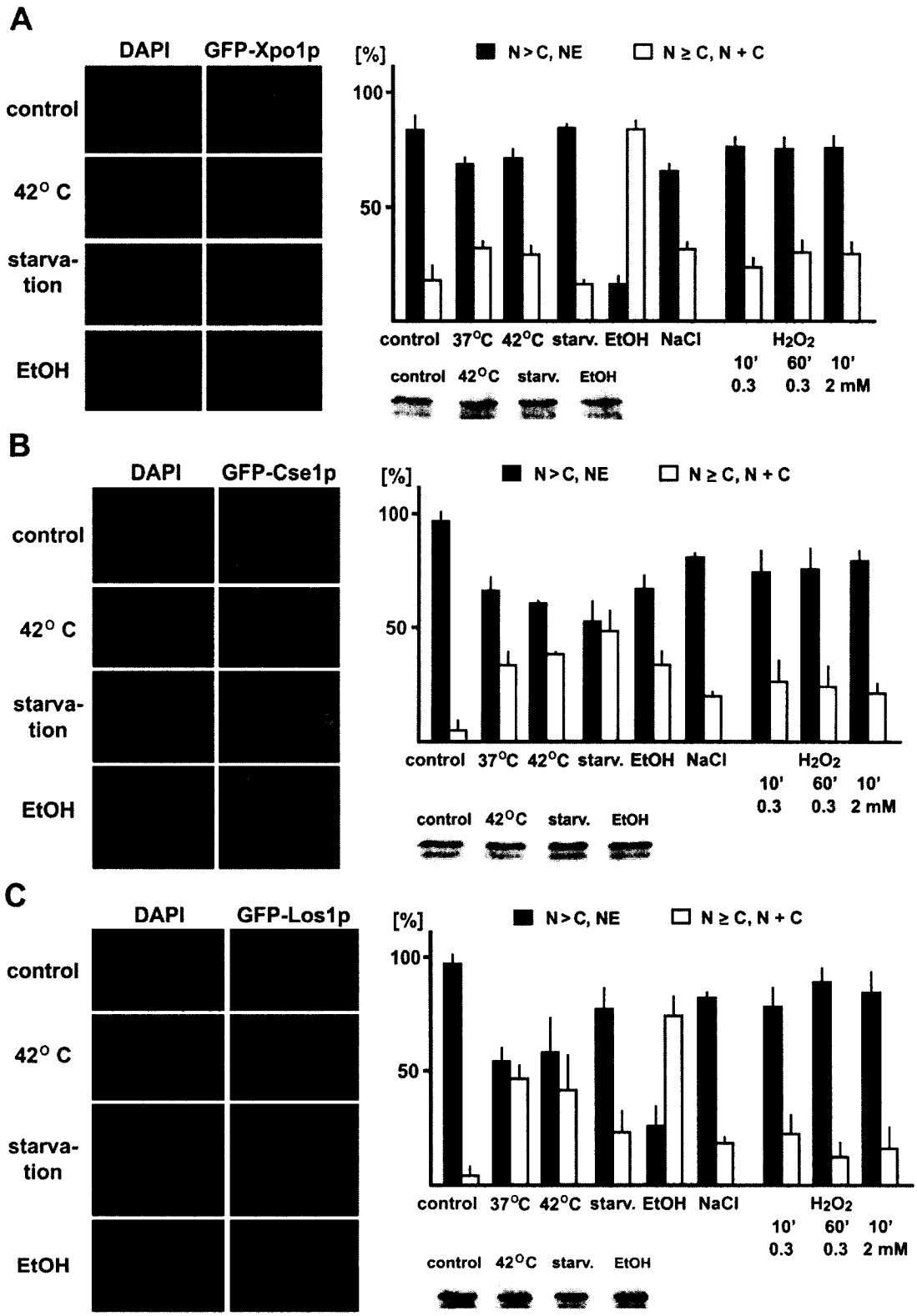

Fig 4.3 Xpo1p, Cse1p and Los1p relocate to the cytoplasm upon stress.

Fig 4.3 Xpo1p, Cse1p and Los1p relocate to the cytoplasm upon stress.

Yeast cells synthesizing GFP-tagged Xpo1p (**A**), Cse1p (**B**) or Los1p (**C**) were exposed to the different stress conditions described in Materials and methods. Carriers associated with nuclei and nuclear envelopes (N>C, NE) or were present in the cytoplasm (N≥C, N+C). Results (means and S.D.) are shown for three independent experiments; a minimum of 100 cells was scored in each experiment. For Western blotting equal amounts of protein from control and stressed cells were separated by SDS-PAGE and nuclear exporters were detected with antibodies against GFP.

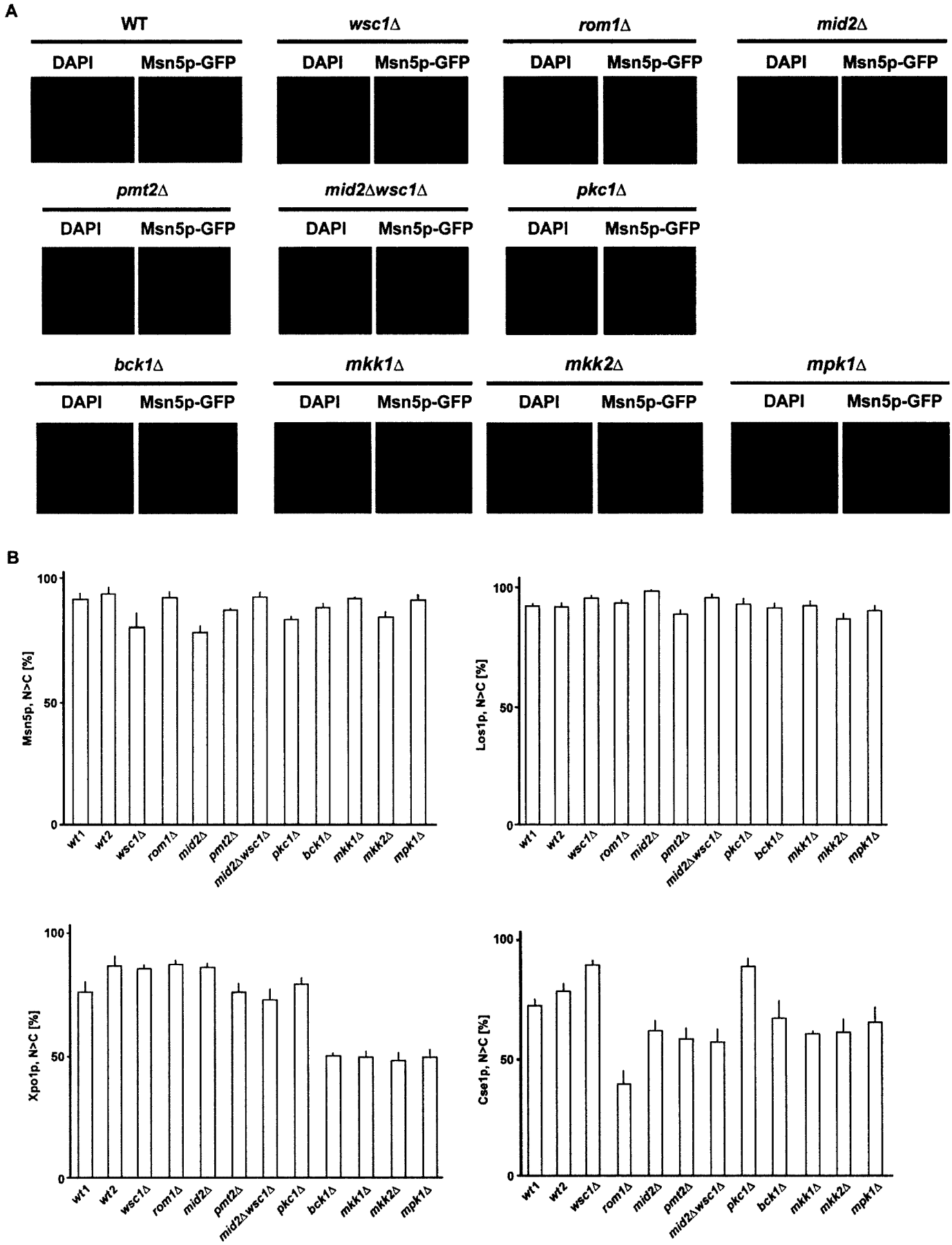

Fig 4.4 The cell integrity MAPK pathway cpntrols the nuclear accumulation of Xpo1p and Cse1p in unstressed cells.

Fig 4.4 The cell integrity MAPK pathway controls the nuclear accumulation of Xpo1p and Cse1p in unstressed cells.

(**A**) GFP-tagged Xpo1p, Cse1p, Los1p and Msn5p were located under non-stress conditions in wild type (wt) and mutant strains, missing different components of the Pkc1p-cell integrity pathway as indicated in the figure. Wt1 is the isogenic wild type for all cells except *mid2Δwsc1Δ* and *pkc1Δ* cells, for which wt2 serves as control. Part (**A**) shows the localization of Msn5p-GFP in different strains, (**B**) the quantification of results obtained for all carriers.

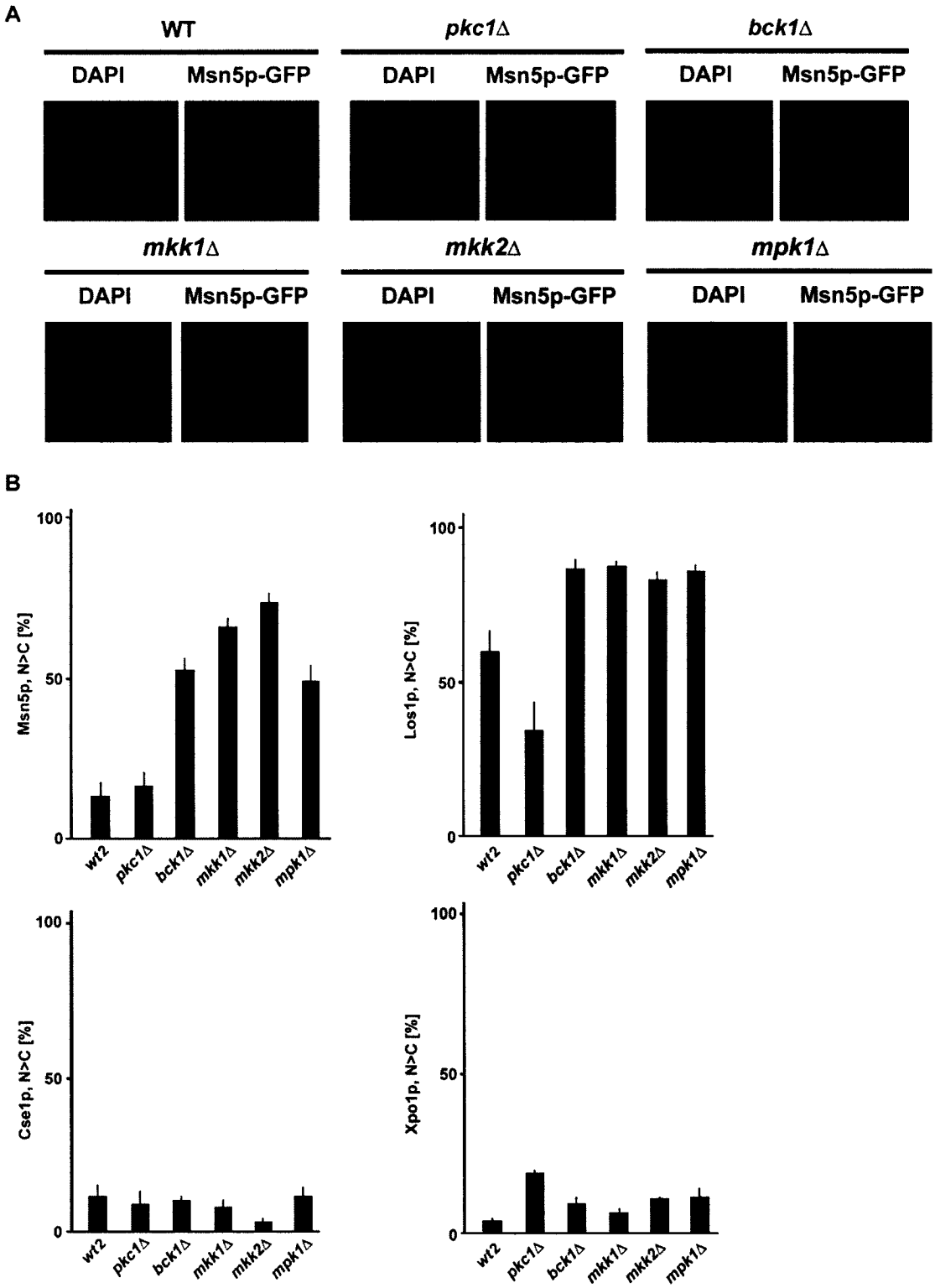

Fig 4.5 Kinases of the cell integrity MAPK module are required to release Msn5p and Los1p from nuclei of ethanol-stressed cells.

Fig 4.5 Kinases of the cell integrity MAPK module are required to release Msn5p and Los1p from nuclei of ethanol-stressed cells.

Wild type and mutant cells synthesizing GFP-tagged exporters were treated for 10 min with 10% ethanol. Cells were immediately fixed and carriers localized by fluorescence microscopy. (**A**) Distribution of Msn5p-GFP in ethanol-stressed wild type and mutant strains. (**B**) Nuclear accumulation of all carriers was quantified in ethanol-treated cells.

CHAPTER 5

General discussion and Summary

5.1 General discussion and summary

5.1.1 Ssa4p redistributes upon stress

We have investigated the effect of certain types of stress on the redistribution of the protein Ssa4p, which is a yeast cytosolic hsp70 family member. In yeast, Ssa4p is present in both the cytoplasm and the nucleus under normal physiological conditions, but accumulates in the nucleus when yeast cells are subjected to certain types of stress, such as ethanol exposure or nutrient starvation. Ssa4p nuclear accumulation is transient when cells are exposed to 10% ethanol. A previous report shows that Ssa4p also responds to starvation by accumulating in the nucleus [Chughtai, 2001]. However, neither mild nor severe osmotic or oxidative stress has any effect on the localization of Ssa4p in yeast (Figure 2.1, Table 2.1).

5.1.2 Regulation of Ssa4p nuclear transport: different signals

Ethanol treatment and starvation result in different mechanisms for Ssa4p nuclear transport; Ssa4p translocates into the nucleus using different import signal sequences. The N-terminal 236 amino acid residues of Ssa4p without the bipartite cNLS promote nuclear accumulation in ethanol exposed cells (Figure 2.1). The short hydrophobic amino acid residues 162 to 171 of Ssa4p, termed the Star-sequence, function as an import signal that allows nuclear transport of the reporter protein in starving cells. However, this Star-sequence does not promote reporter protein nuclear accumulation upon ethanol stress (Figure 2.3 and Chughtai, 2001). The Ssa4p export signal is located within its N-terminal 236 amino acid residues

and mediates Ssa4p nuclear export during recovery from ethanol stress (Figure 2.4 and Figure 3.6). This Ssa4p export signal is different from the classical leucine-rich NES which is also in the N-terminal domain of Ssa4p. NES containing protein nuclear export is normally mediated by the carrier protein Xpo1p, but Ssa4p(1-236)-GFP transport from nucleus to cytoplasm neither requires Xpo1p nor is inhibited by LMB. This suggests that the N-terminal domain of Ssa4p contains more than one nuclear import and export signal sequence, which promotes Ssa4p nuclear transport in response to different physiological conditions.

5.1.3 Regulation of Ssa4p nuclear transport: specific transport pathways and unique carriers

We investigated the cytoplasmic components that are required for Ssa4p nuclear transport during and after stress treatment. We demonstrated that Ssa4p uses specific transport pathways and unique carriers to mediate its nuclear import/export upon ethanol exposure and recovery.

First, the classical import pathway is blocked in ethanol stressed cells and the NES/Xpo1p mediated export is not essential for Ssa4p translocation from the nucleus to the cytoplasm during recovery from ethanol treatment.

Second, the transport adapter importin α is not required for Ssa4p nuclear import, which is in line with the fact that importin α specifically binds to cNLS (Figure 2.1, 2.2, 2.5 and Figure 3.12).

Third, the importin β family member, Kap95p, which normally interacts with importin α, is not required for Ssa4p nuclear import upon ethanol stress. As

shown in Figure 2.2, 2.6 and 2.9, the importin β family member, Nmd5p, is a nuclear import carrier for Ssa4p. The protein interaction assay in growing yeast cells provides evidence that Nmd5p and Ssa4p form a genuine import complex which can be dissociated by GTP bound Gsp1p. During the recovery stage from ethanol treatment, Ssa4p nuclear export is mediated by its export carrier Msn5p. We have presented direct evidence of the association between Ssa4p and Msn5p both *in vitro* and *in vivo* (Figure 3.1 and 3.3). These results imply that cells can regulate protein transport using different transport components. This allows cells to adjust efficiently when exposed to environmental stresses.

5.1.4 Regulation of Ssa4p nuclear transport: localization of carrier proteins

The carrier's subcellular location is one of the factors involved in the regulation of Ssa4p nuclear transport. We demonstrate a correlation between the mislocalized cytoplasmic Msn5p and the increased nuclear accumulation of Ssa4p (Figure 3.6). This implies that the abnormally localized carrier protein Msn5p may slow down its cargo's transport through the NPC and thus regulate the specific nuclear trafficking process. One of the possible mechanisms could be an inhibition of the interaction between Msn5p and Ssa4p. Evidence presented in chapter 3 also indirectly supports the idea that carrier localization can regulate its cargo's transport. Pho4p and Far1p are other cargos requiring Msn5p as their exporter. Although the mechanisms for the Msn5p-mediated nuclear export of these two proteins is more complicated than Ssa4p, our results show that upon certain types of stress Msn5p mislocates to the cytoplasm and these two proteins redistribute partially from the nuclei to the cytoplasm (Figure 3.9). This implies

that the localization change for carrier Msn5p could be one of the regulation factors for its cargo's intracellular distribution.

5.1.5 Regulation of Ssa4p nuclear transport: stress

The regulation of Ssa4p nuclear transport is not only limited to the components required for its trafficking. Stress itself regulates Ssa4p nuclear transport.

In chapter 2, we have identified two distinct nuclear import steps that are modulated by ethanol. First, the formation of the import complex Ssa4p/Nmd5p is increased when cells are exposed to ethanol (Figure 2.9). Second, the docking of Nmd5p at the NPC is elevated in ethanol-treated cells (Figure 2.10). These changes lead to an increased concentration of Ssa4p in the nuclei, suggesting that ethanol can up-regulate Ssa4p nuclear import.

In chapter 3 we show that nutrition limitations affect Ssa4p nuclear export by regulating the intracellular localization of the carrier protein Msn5p. When cells grow on non-fermentable carbon sources, Msn5p mislocates from the nucleus to the cytoplasm resulting in an elevated level of Ssa4p (Figure 3.7). Various reports have shown that other types of stress also affect protein nuclear transport [Hirano, 2006].

This thesis shows that stresses regulate Ssa4p nuclear export through two effects. The first is a redistribution of the carrier Msn5p (Figure 3.4). Msn5p is sensitive to several forms of stress and results in the partial inhibition of Ssa4p nuclear export. The second effect is on the regulation of the formation of the export complex Ssa4p/Msn5p (Figure 3.5). Several types of stress that relocate the

carrier Msn5p can reduce the interaction of Msn5p with Ssa4p. In fact, Ssa4p is not the only protein for which nuclear transport is regulated by stresses. We showed that the localization of Far1p and Pho4p are both sensitive to stress (Figure 3.9).

Stress is, however, only one of the multiple regulators for protein nuclear transport. As an example, Msn5p-mediated Mig1p nuclear export occurs only upon starvation but not under heat or ethanol stress, although Msn5p itself mislocates upon heat and ethanol treatment (Figure 3.8). This suggests that Mig1p might use different export carrier for its transport upon different stresses.

5.1.6 Regulation of Ssa4p nuclear transport: kinase pathways

Stresses also regulate Ssa4p nuclear trafficking by activating the kinase pathways. Ethanol treatment can affect plasma membrane fluidity and activate the protein kinase C (Pkc1p) cell integrity pathway. Our data shows that Ssa4p nuclear import upon ethanol stress requires Pkc1p (Figure 2.11). MAPK is the major downstream pathway of PKC. Figure 4.5 and 4.6 show that the distribution of nuclear exporters is regulated by the MAPK module.

5.1.7 Regulation of Ssa4p nuclear transport: Snf1 kinase

Nutrient limitation creates an energy shortage and affects nuclear transport, since most of the protein trafficking pathways are energy-dependent. In chapter 3 and 4, we demonstrated that when cells are growing in non-fermentable carbon media, all four yeast nuclear exporters, Msn5p, Cse1p, Xpo1p and Cse1p, relocate to the cytoplasm (Figure 4.1). Similar results were observed in cells

treated with rapamycin, an inhibiter of the TOR kinase pathway (Figure 4.2). That pathway is at the center of the cell metabolism and growth regulation, and is activated by nutrient availability. Kinase Snf1 is known to be activated in response to glucose depletion, nitrogen limitation, heat and osmotic stress. We have demonstrated that the localizations of yeast intercellular nuclear exporters are also regulated by Snf1 kinase (Figure 4.1). The possible mechanisms for kinase regulated protein nuclear translocation could be 1) affecting the transport carrier's proper localization, 2) other modifications, which remains to be elucidated.

5.1.8 Hsp70 and cell survival

Hsp70 are important for cell viability. Our investigations present further evidences of the protective role of Ssa4p under various types of stresses. Figure 3.11 shows that the enhanced synthesis of Ssa4p can rescue cells from stress-induced death and increase the survival ratio. Although we observed some increased stress survival from the expression of the N-terminal *SSA4(1-236)* gene, it is not statistically significant when compared to the full length *SSA4*. These results indicate that the molecular chaperone function of Hsp70 Ssa4p is important for protection against environmental stresses.

5.1.9 Transport carriers and cell survival

Interestingly, not only Hsp70 Ssa4p, but also the nuclear transport carriers Nmd5p and Msn5p play a role in cell reaction to a variety of stresses. Although *NMD5* and *MSN5* are not essential genes for cell viability, the survival rates of

mutant *NMD5Δ* and *MSN5Δ* are lower than wild type cells upon heat, ethanol, starvation and oxidative stress (Figure 3.10). This suggests that the nuclear transport of many proteins in response to various stresses are interrupted without the carriers Nmd5p and Msn5p. Importin β-mediated protein transport could be another way to regulate cell survival. *NMD5* deletion mutant strain is viable but shows mislocalization of TFIIS and Hog1p. Both proteins are involved in gene transcription. Hog1p also functions as a MAP kinase in response to osmotic stress. It has not been reported that *MSN5* deletion affects its cargos' intracellular distribution, but our data show that several forms of stresses, which induce the mislocalization of Msn5p, also can cause its substrates Pho4p and Far1p to redistribute from the nucleus to the cytoplasm (Figure 3.9). Both proteins play a role in cell metabolism and stress response. Their cellular distribution might be important to their normal functions.

5.1.10 Summary

In summary, this thesis presents the following new findings related to the mechanisms of Hsp70 nuclear trafficking upon stress and the responses of transport carriers to various physiological states.

1. We show for the first time that in yeast, cytoplasmic Hsp70 Ssa4p specifically accumulates in the nuclei upon ethanol stress. Its N-terminal domain 236 amino acid residues are sufficient for nuclear import.
2. This is the first report that identifies Nmd5p as an Ssa4p nuclear importer upon ethanol exposure and Msn5p as an Ssa4p nuclear exporter during

recovery from ethanol exposure. We also show for the first time that Ssa4p and Msn5p can interact directly *in vitro*.

3. We show that Pkc1p and the sensors of the cell integrity signaling pathway are involved in ethanol-induced Ssa4p nuclear accumulation.
4. We demonstrate for the first time that ethanol stress regulates Hsp70 Ssa4p nuclear import by affecting two key steps: 1) the formation of import complex Ssa4p/Nmd5p, which is enhanced 2) the docking of Nmd5p to NPCs which is also enhanced.
5. We show that the localization of all four exporters in yeast is regulated by Snf1 kinase, nutrition supply and stress. Different exporters have distinct sensitivities to specific stress forms.
6. This is the first report showing that stress regulates Msn5-mediated nuclear export by interfering with the formation of export complexes.

Many new cargos, carriers and specific transport pathways will undoubtedly be identified in the future. These new insights will provide a better understanding of nucleocytoplasmic exchange and cell adjustments to different physiological and pathological conditions. We hope this thesis has helped to understand better some aspects of Hsp70 transport under stress and laid the foundation for further study of its cytoprotective role when cell are exposed to environmental insults.

5.2 Hsp70 and nuclear transport contribution to disease therapy

Research on the mechanisms of Hsp70 nuclear trafficking could lead to new diagnostic indexes and therapeutic strategies for human diseases. As an example, the clinical symptoms of West Nile virus illnesses are due to cell apoptosis induced by a West Nile virus capsid protein. Crm1p-mediated nuclear export of this capsid protein could contribute to its degradation and thus could provide a novel therapeutic treatment for West Nile virus diseases [Oh, 2006].

Another example is oculopharyngeal muscular dystrophy, which is a dominant autosomal disease whereby patients have progressive difficulty in swallowing and eyelids elevation. The major cause of this disease is the nuclear inclusions formed by the aggregation of some proteins. Increasing the expression level of *HSP70* and its nuclear accumulation can significantly reduce protein aggregates and cell death [Wang, 2005].

Recent studies have been shown that the HSP70 overexpression in malignant tumors of different origins is related to abnormal cell proliferation, poor differentiation and lymph node metastases resulting in poor therapeutic outcome [Rohde, 2005]. The possible mechanisms for Hsp70 tumorigenesis could be that it promotes cancer cell survival and inhibits cell death induced by cancer-related hypoxia, inflammatory cytokines and anticancer drugs. Using antisense HSP70 oligonucleotides or adenovirus expressing antisense Hsp70 cDNA, which depletes Hsp70, selectively kills cancer cells in both cancer cell culture and tumor

xenografts of glioblastoma, breast and colon carcinoma in mice [Rohde, 2005; Nylandsted, 2002].

Studies on Hsp70 and its intracellular transport could very well provide wider and deeper insight on various diseases and the exploration of new therapeutic ideas and agents.

5.3 Future considerations

The findings of this thesis suggest several directions for further research. We demonstrated that 1) protein kinase C is involved in Ssa4p nuclear import upon ethanol stress (chapter 2); and 2) that the Snf1 kinase and the MAPK pathway are required for the localization of the nuclear exporter Msn5p under normal and stress conditions (chapter 4). Unpublished data also showed that Ssa4p nuclear import under stress condition is dependent on Snf1 kinase. The above evidence indicates that protein modification by phosphorylation may play a role in Ssa4p stress-induced nuclear transport. It would be interesting to define which steps or components are required for Ssa4p nuclear trafficking and how they are affected by phosphorylation. Is there a modification of the carrier, cargo or Nups that regulates Ssa4p nuclear transport upon stress? Which kinases are involved in the process? Furthermore, by screening the probable phosphorylation sites of proteins and using site-directed mutagenesis assay, it could be possible to identify the key modification sites for Ssa4p nuclear transport.

Another important issue that needs to be addressed is how Hsp70 assists accelerated recovery from stress following it transport into the nucleus. One possible explanation is that the accumulated nuclear Hsp70 might be required for nuclear proteins (such as histone) to restore their normal functions. The other function of Hsp70 in the nucleus could be its interactions with transcription factors and therefore regulating gene expression in response to stress conditions. Another possibility is that Hsp70 might be required in the maintenance of nuclear organization. It has been shown that mammalian Hsc70 is retained in the nucleus upon stress exposure and associates with fibrillarin and ribosomal protein rpS6, proteins composed of nucleoli and the small ribosomal subunits [Kodiha, 2005].

Our data demonstrates that stress, nutrition sources and the PKC-MAPK pathway which normally activate many transcription factors in the nucleus are involved in the regulation of Ssa4p nuclear transport. The relationship between nuclear Hsp70 and transcription factors contributing to the cell metabolism regulation needs to be explored.

In addition, it would be of interest to investigate if other heat shock protein family members and their cochaperones are involved in Hsp70 nuclear transport upon stress. Evidences show that Hsp90 and its cochaperones Sti1 and Cpr7 interact with Hsp104 in response to altered metabolic conditions in yeast [Abbas-Terki, 2001]. Hsp90 forms a heterocomplex with Hsp70, Hop, Hsp40 and regulates its substrates function and trafficking, including steroid receptors, protein kinases and transcription factors [Pratt, 2003]. Investigating the effects of

other heat shock protein family members on Hsp70 nuclear transport would help us understand better how cell adjusts to different physiological conditions.

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APPENDIX

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[1]

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SPECIFIC AIMS

Cytoplasmic proteins of the heat shock protein (hsp)70 family, such as Ssa4p in *Saccharomyces cerevisiae*, accumulate in the nuclei in response to certain forms of stress. With the experiments described here, we have defined the signal and the molecular mechanisms that mediate the nuclear concentration of Ssa4p in response to ethanol exposure.

PRINCIPAL FINDINGS

1. The cytoplasmic hsp70 Ssa4p specifically accumulates in the nuclei upon incubation with ethanol

Various types of stress may alter the nucleocytoplasmic distribution of proteins. Ssa4p, cytoplasmic and nuclear under normal physiological conditions, accumulates in the nuclei when cells have been treated with ethanol. By contrast, the classical nuclear transport pathway is inhibited by ethanol (**Fig. 1***A, C*). Unlike ethanol treatment, osmotic or oxidative stress fails to concentrate Ssa4p in the nuclei.

2. The N-terminal domain of Ssa4p is sufficient to concentrate Ssa4p in the nuclei

Ssa4p contains a segment with clusters of basic amino acid residues that is similar to a classical nuclear localization sequence (cNLS). However, this potential NLS is not required for ethanol-induced nuclear localization. An N-terminal segment of 236 amino acid residues of Ssa4p fused to GFP, termed Ssa4p(1–236)–GFP, is lacking the potential cNLS but mediates the nuclear accumulation of the non-nuclear reporter protein GFP (Fig. 1*B*). These results are in line with the hypothesis that a nonclassical signal and transport route is used for Ssa4p nuclear import.

3. Different targeting signals are used to accumulate Ssa4p in ethanol stressed and starving cells

Our previous studies have shown that a short, hydrophobic sequence in the N-terminal domain of Ssa4p, termed Star (for starvation), promotes targeting to the nucleus of starving cells when fused to β-galactosidase. By contrast, Star–β-galactosidase does not concentrate in the nuclei in response to ethanol treatment. These results suggest that Ssa4p contains more than one signal for nuclear import and that distinct signals are used when cells have been exposed to different forms of stress.

4. Nuclear accumulation of Ssa4p–GFP is reversible

The concentration of Ssa4p in the nuclei of stressed cells is only transient. Upon removal of the stressor ethanol, Ssa4p(1–236)–GFP relocates to the cytoplasm, suggesting that this protein shuttles between both compartments.

5. Nuclear accumulation of Ssa4p in ethanol-treated cells requires the Ran/Gsp1p GTPase cycle

Different conditional lethal mutants deficient in distinct nuclear transport factors were analyzed for their capacity to import the Ssa4p N-terminal domain into nuclei upon exposure to ethanol. These studies revealed that Ran/Gsp1p and the GTPase-interacting factors Rna1p (GTPase-activating protein) and Prp20p (guanine nucleotide exchange factor) are necessary for Ssa4p nuclear import in stressed cells.

6. Classical nuclear import is not required to accumulate Ssa4p in the nuclei of stressed cells

Classical nuclear import relies on the Ran GTPase system and the dimeric import receptor importin-α/importin-β. This import route is involved in nuclear

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.03-0947fje; doi: 10.1096/fj.03-0947fje

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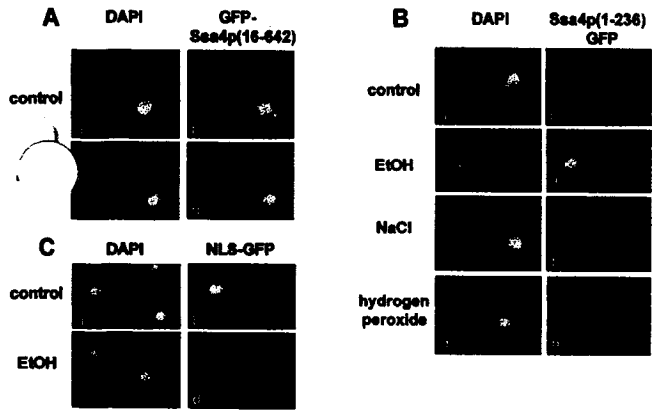

Figure 1. Stress-induced relocation of the cytoplasmic hsp70 Ssa4p and NLS–green fluorescent protein (GFP). Wild-type yeast cells synthesizing Ssa4p fusion proteins or NLS–GFP were grown overnight in drop-out medium containing 2% galactose. In fixed cells, DNA was visualized with 4′,6-diamidino-2-phenylindole (DAPI), and fusion proteins were localized by fluorescence microscopy. The experiment was performed with (*A*) GFP–Ssa4p(16–642), (*B*) Ssa4p(1–236)–GFP, and (*C*) NLS–GFP. GFP-containing reporter proteins were located in control cells (*b*) or after 10 min exposure to 10% ethanol (EtOH; *d*), 0.4 M sodium chloride (NaCl; *f*), or 0.3 mM hydrogen peroxide (*h*).

trafficking of a wide variety of substrates that contain a cNLS. The classical nuclear import pathway is inhibited when cells have been treated with ethanol, making transport of Ssa4p(1–236)–GFP via importin-α/importin-β unlikely. In line with this hypothesis, we demonstrate that Srp1p, yeast importin-α, is not essential to concentrate the reporter protein in the nuclei of ethanol-stressed cells.

7. The nuclear carrier Nmd5p concentrates Ssa4p in the nuclei after incubation with ethanol

The requirement of the Ran/Gsp1p GTPase cycle for Ssa4p nuclear accumulation suggested that a member of the importin-β family of carriers plays a role in Ssa4p nuclear import. Indeed, the analysis of mutants inactivated for different importin-β genes shows that the carrier Nmd5p promotes the nonclassical nuclear accumulation of Ssa4p and its N-terminal domain in ethanol-stressed cells (**Fig. 2*A–C***). As such, various mutant strains that contain the wild-type *NMD5* gene rapidly concentrate Ssa4p(1–236)–GFP in the nuclei. By contrast, mutant cells carrying a knockout of *NMD5* fail to accumulate Ssa4p in the nuclei (Fig. 2*A–C*).

8. The formation of Ssa4p(1–236)–GFP/Nmd5p import complexes is increased by ethanol

The first step of nuclear import is the generation of the cargo/carrier complex. Ssa4p(1–236)–GFP associates with Nmd5p, and this interaction can be dissociated by Gsp1p–GTP, indicating that Ssa4p(1–236)–GFP and Nmd5p are together part of a bona fide import complex. Although Ssa4p(1–236)–GFP can bind to Nmd5p in unstressed cells, the formation of Ssa4p(1–236)–GFP/Nmd5p import complexes is significantly increased by exposure to ethanol.

9. Binding of the carrier Nmd5p to nuclear pore complexes (NPC) is stimulated by ethanol

Following the formation of import complexes, nuclear carriers will dock at the nuclear pore to promote the translocation of its cargo. Nmd5p binding to nuclear

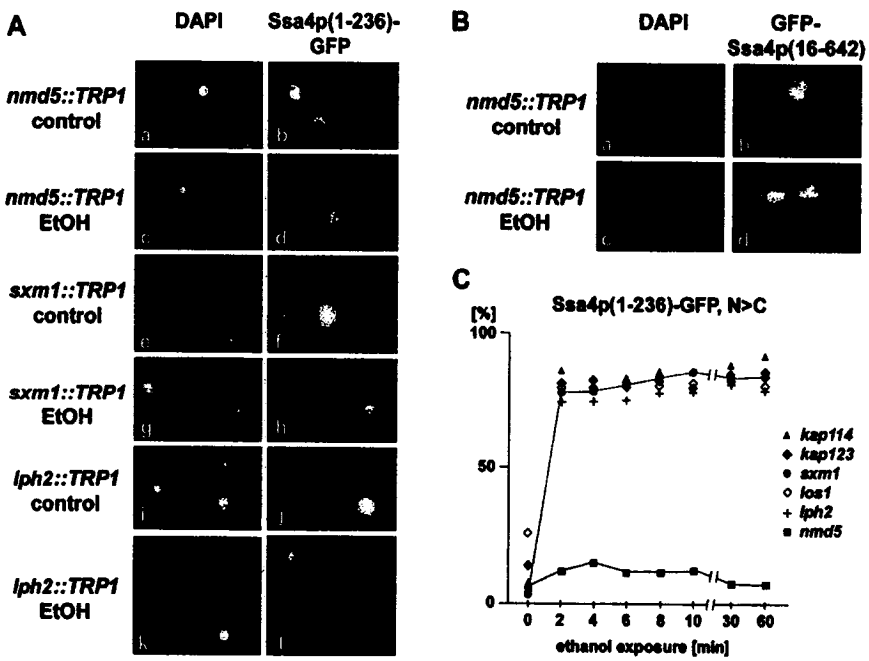

Figure 2. Ethanol-induced nuclear accumulation of Ssa4p(1–236) requires the carrier Nmd5p. (*A*)Yeast strains carrying a mutation in one of the importin-β genes were analyzed for the nuclear accumulation of Ssa4p(1–236) in response to ethanol stress. The distribution of Ssa4p(1–236)–GFP is shown for strains *nmd5::TRP1*, *sxm1::TRP1*, and *lph2::TRP1*. Control and ethanol-treated cells were analyzed in parallel. (*B*) GFP–Ssa4p(16–642) was located in *nmd5::TRP1* control and ethanol-stressed cells as indicated. (*C*) Ethanol-induced nuclear accumulation of Ssa4p(1–236)–GFP in mutant yeast strains deleted for one of the importin-β genes. Mutant cells synthesizing Ssa4p(1–236)–GFP were stressed with 10% ethanol for the time indicated. Cells were fixed, and nuclear accumulation (N>C) of the fusion protein was monitored by fluorescence microscopy. Results were observed at least three times. Nuclear accumulation of Ssa4p(1–[illegible]–GFP was determined at the times [illegible]d, 100 cells were scored in each independent experiment for every time point, and means are depicted in the figure.

pore complexes is regulated by ethanol, and it is significantly enhanced in cells that have been exposed to this alcohol.

10. Ethanol-induced concentration of Ssa4p in the nuclei requires protein kinase C (Pkc1p) and sensors of the cell integrity signaling pathway

Nuclear accumulation of Ssa4p(1–236)–GFP was tested in strains deleted for the single gene, which encodes Pkc1p in budding yeast. This mutant is unable to concentrate the reporter protein in the nuclei when cells have been exposed to ethanol. *MID2* and *WSC1* code for proteins located in the plasma membrane; they act as sensors of cell integrity and can induce the activation of Pkc1p. The deletion of *MID2* or *WSC1* significantly reduces the nuclear accumulation of Ssa4p(1–236)–GFP in stressed cells, suggesting a link between Pkc1p activation and nuclear import of Ssa4p.

CONCLUSIONS AND SIGNIFICANCE

The appropriate response to stress is crucial to cell survival and recovery from various insults. As such, the nuclear accumulation of proteins of the hsp70/hsc70 family plays a critical role in the repair of stress-induced injuries. Nuclear proteins may be particularly vulnerable to damage by ethanol, therefore requiring the nuclear accumulation of heat shock protein to restore cellular functions. Different mechanisms may induce the concentration of a protein in the nucleus. These could include an increase in nuclear import, inhibition of nuclear export, as well as changes in nuclear or cytoplasmic retention. Our studies suggest that the up-regulation of Ssa4p nuclear import contributes to its concentration in the nuclei. We show that a nonclassical transport route which requires Nmd5p, a member of the importin-β family of nuclear carriers, mediates Ssa4p nuclear import in ethanol-treated cells. For this transport pathway, we have identified two distinct steps that are modulated by stress. First, the formation of import complexes containing Ssa4p(1–236)–GFP and Nmd5p is increased in ethanol-treated cells. Second, docking of the carrier Nmd5p at the nuclear pore complex is enhanced when cells have been incubated with ethanol. Both changes in Ssa4p nuclear trafficking can be expected to promote the concentration of the heat shock protein in the nuclei (summarized in **Fig. 3**). The regulation of hsp70 nuclear transport on

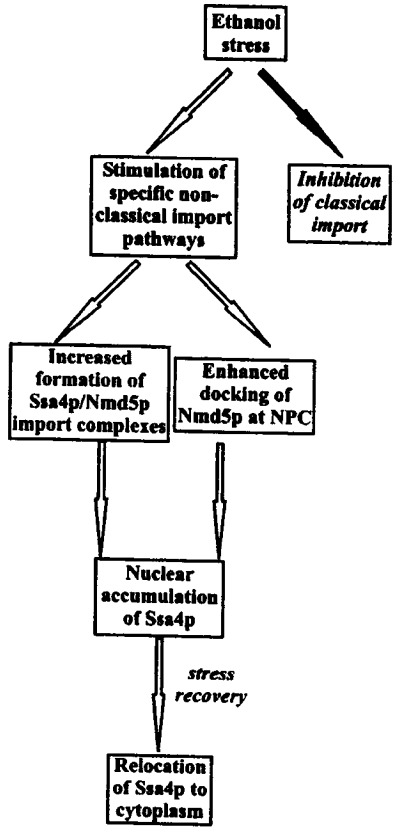

Figure 3. Concentration of Ssa4p in the nuclei upon exposure to ethanol stress. Simplified model for the nuclear accumulation of the hsp70 Ssa4p in cells that have been treated with ethanol. See text for details.

different levels of the import reaction may help cells to fine-tune the efficiency of the nuclear accumulation according to the severity of stress and the physiological requirements of the cell.

In addition to identifying the nuclear transport pathway for Ssa4p, our results show that Pkc1p plays an essential role in nuclear trafficking of the heat shock protein. Moreover, Mid2p and Wsc1p, sensors of the cell-integrity pathway, which are known to be upstream activators of Pkc1p, are necessary to concentrate Ssa4p(1–236)–GFP in the nuclei of ethanol-treated cells. We propose that Mid2p and Wsc1p sense changes in the membrane fluidity induced by ethanol, leading to the activation of Pkc1p and ultimately to the nuclear accumulation of Ssa4p. Future experiments will have to define the molecular reactions that link Pkc1p activation to the concentration of Ssa4p in the nuclei.

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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

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ABSTRACT

Cytoplasmic proteins of the hsp70/hsc70 family redistribute in cells that have been exposed to stress. As such, the hsp70 Ssa4p of the budding yeast *S. cerevisiae* accumulates in nuclei when cells are treated with ethanol, whereas classical nuclear import is inhibited under these conditions. The N-terminal domain of Ssa4p, which is lacking a classical NLS, mediates nuclear accumulation upon ethanol exposure. Concentration of the Ssa4p N-terminal segment in nuclei is reversible, as the protein relocates to the cytoplasm when cells recover. Mutant analysis demonstrates that the small GTPase Gsp1p and GTPase-modulating factors are required to accumulate Ssa4p in nuclei upon ethanol stress. Moreover, we have identified the importin-β family member Nmd5p as the nuclear carrier for Ssa4p. Ethanol treatment significantly increases the formation of import complexes containing Nmd5p and the N-terminal Ssa4p domain. Likewise, docking of the carrier Nmd5p at the nuclear pore is enhanced by ethanol. Furthermore, we show that the stressed-induced nuclear accumulation of Ssa4p depends on signaling through protein kinase C and requires sensors of the cell integrity pathway.

Key words: Stress • heat shock proteins • nuclear transport • PKC

Members of the hsp70 family of proteins are involved in a variety of cellular processes, including the targeting of polypeptides to different organelles, such as the nucleus (1–3). In addition, hsp70s are implicated in repair of cell and tissue damage in response to heat shock and other forms of insults (reviewed in 4–6). The yeast *Saccharomyces cerevisiae* contains six cytoplasmic hsp70s, which are members of two families. The first family consists of Ssa1p, Ssa2p, Ssa3p, and Ssa4p; proteins Ssb1p and Ssb2p are members of the second family. Gene products of the *SSA* family are highly homologous; however, their expression is regulated differently. Proteins Ssa1p and Ssa2p are produced under normal growth conditions; upon heat shock, *SSA1* gene expression increases, and the synthesis of Ssa3p and Ssa4p is highly induced (reviewed in 7). Like heat stress, ethanol concentrations above 4–6% lead to a strong induction of heat shock protein synthesis (reviewed in 8). In response to stress, some cytoplasmic hsp70s accumulate in nuclei, whereas nuclear import of many other proteins and nuclear export of bulk polyA$^+$ RNA is inhibited (9–12). Export of mRNA encoding Ssa4p in stressed yeast cells has been studied in some detail (10, 13), whereas the molecular mechanisms underlying nuclear targeting of hsp70s are poorly defined. Rat hsc70, when injected into

Xenopus oocytes, shuttles between the nucleus and the cytosol (14). The classical bipartite NLS (cNLS) of hsc70 (15, reviewed in 16), located in the N-terminal domain, is believed to play the major role for nuclear import under normal growth conditions. However, partial deletion of the cNLS does not prevent rat hsc70 import into nuclei of *Xenopus* oocytes in unstressed cells. On the basis of these data, it was concluded that a nonclassical nuclear transport signal is present in hsc70s that promotes nuclear targeting by a pathway distinct from cNLS-mediated nuclear transport (17). Not all cytoplasmic hsp70s, however, are able to concentrate in nuclei. For instance, Ssb1p nuclear accumulation is prevented by a nuclear export signal at the C-terminal end of the protein (18).

Although previous studies with rat hsc70 have shown that the bipartite cNLS is not required for nuclear entry, these experiments did not address nuclear transport in stressed cells. Furthermore, it has not been analyzed whether a signal distinct from the cNLS mediates stress-induced nuclear transport. Likewise, it was unclear whether nuclear transport mediated by this nonclassical NLS uses components of the classical nuclear import pathway. Nuclear transport via cNLSs has been characterized in depth during the past years (reviewed in 19, 20). The consensus model proposes that the cNLS is recognized by the dimeric cNLS-receptor importin-$\alpha/\beta 1$ and subsequently translocated across the nuclear pore complex (NPC). Srp1p, the yeast homologue of importin-α, is essential for classical nuclear import, and several soluble factors are required as well. In *S. cerevisiae*, these factors include the predominantly nuclear GTPase Gsp1p, the cytoplasmic GTPase-activating protein Rna1p, and the guanine nucleotide exchange factor Prp20p in the nucleus. *GSP1*, *RNA1*, and *PRP20* are essential components, and mutations in these genes can interfere with nuclear import of cNLS-containing proteins (reviewed in 21).

Nuclear export of bulk polyA$^+$ mRNA and import of cNLS-containing polypeptides is sensitive to ethanol (10, 12, 13). Likewise, a variety of other stress conditions interfere with classical nuclear trafficking (12). Stress-induced inhibition of classical transport is associated with a redistribution of soluble nuclear transport factors. In particular, the small GTPase Gsp1p, accumulated in nuclei under normal conditions, equilibrates between nucleus and cytoplasm in response to ethanol, starvation, heat stress, and hydrogen peroxide (12).

Different forms of stress can activate distinct signaling pathways in *S. cerevisiae* (22–24). In budding yeast, protein kinase C (Pkc1p) plays a key role in maintaining cellular integrity (reviewed in 22, 25), and the single *PKC1* gene is crucial for signaling through the cell integrity pathway (reviewed in 26). Pkc1p-activated pathways are not only involved in remodeling of the cell wall during growth, they also play a role in the mating response and cell cycle progression. With respect to cell integrity, it was proposed that changes in membrane fluidity could be involved in the activation of Pkc1p (27). Furthermore, Pkc1p was also shown to regulate the relocation of nuclear proteins to the cytoplasm when cells have been exposed to high osmolarity (28). Several cellular components can induce Pkc1p activation; this includes proteins Wsc1p (also known as Hcs77p and Slg1p) and Mid2p, sensors of cell integrity, which both reside in the cytoplasmic membrane (25, 29–31). The activity of Mid2p as a sensor depends on Pmt2p, an enzyme located in the ER that is involved in Mid2p O-glycosylation (31, 32). A deletion of *PMT2* interferes with Mid2p glycosylation and signaling of the sensor to Pkc1p (32).

It has not been analyzed previously whether nucleocytoplasmic trafficking of heat shock proteins can be regulated by any of the components that respond to changes in cell integrity. To analyze this question, we have focused on the yeast hsp70 Ssa4p and characterized its nuclear transport

route in ethanol-stressed cells. We demonstrate that Ssa4p nuclear accumulation upon stress exposure requires the nuclear carrier Nmd5p, sensors of cell integrity pathway, and Pkc1p.

MATERIALS AND METHODS

Yeast strains, media, and transformation

The following yeast strains were used: RS453 (MATa *ade2 leu2 ura3 trp1 his3*), SS328 (MATa *ade2-101 his3Δ200 lys2-801 ura3-52*), PRP20/2A (MATa *prp20-1 ade2-101 his3Δ200 lys2-801 ura3-52*), L4884 (MATa *SRP1 can1-100 ura3-1 leu2-3,112 his3-11,15 ade2-1 trp1Δ63*), L5677 (MATa *srp1-31 can1-100 ura3-1 leu2-3,112 his3-11,15 ade2-1 trp1Δ63*), SJ21R-35-4 (MATa *RNA1 ura3 leu2 ade1 rnh::URA3*), SJ21R-6-3 (MATa *rna1-1 ura3 leu2 ade1 rnh::URA3*), PSY962 (MATa *gsp1::HIS3 gsp2::HIS3 ura3-52 leu2Δ1 trp1-63* pCEN *URA3 GSP1*), Y-547 (MATα *los1::HIS3 trp1 leu2 ade2 ura3 lys1 his3*). Strains carrying mutations in one of the importin-β genes or NPC clustering strains have been described previously (33, 34). Yeast cells mutated in components of the cell integrity pathway were kindly provided by H. Bussey (Montreal). Cells were transformed and selected on drop-out media according to standard protocols. Expression of fusion genes was induced by overnight growth in selective medium with 2% galactose. The growth media of yeast strains deficient in one of the components of the cell integrity pathway were supplemented with 10% sorbitol.

Plasmid constructions

DNA manipulations carried out in *E. coli* strain XL1-Blue were verified by sequencing. The Mut1 version of GFP was used to generate Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) (35). Fusion genes were cloned into centromeric plasmids, which carried the *URA3* or *LEU2* marker and the inducible *GAL1* promoter. A bacterial expression plasmid encoding His_6-tagged Nmd5p was provided by G. Schlenstedt (Homburg). The His_6-Nmd5p coding sequence was cloned into a yeast expression vector carrying the *GAL5* promoter (36).

Plasmid shuffling

The mutant allele *gsp1-2*, encoded by a centromeric plasmid carrying the *TRP1* marker, was introduced into strain PSY962 (37) following standard procedures. For comparison, wild-type *GSP1* was expressed from the same plasmid vector.

Western blot analysis and quantitation of ECL signals

Proteins were detected by Western blotting with antibodies against GFP (diluted 1:200, Clontech, Palo Alto, CA) or the His_6-epitope (1:100, Santa Cruz Biotechnology, CA) as described (38). Signals obtained after ECL (Pierce, Rockford, IL) were quantified by densitometry of the films using the software "Spot Density Tools" as suggested by Alpha Innotech Corporation (San Leandro, CA). In brief, density measurements were carried out for each area of interest and corrected for background. All of the samples quantified for an individual experiment were present on the same filter. Filters were exposed to film for different times to ensure that the strength of the brightest signal was below the saturation level of the film.

Fluorescence microscopy of yeast cells

Yeast cells were grown and exposed to stress as detailed in the text. Cells were fixed with 3.7% formaldehyde for 10 min at room temperature, washed in 0.1 M potassium phosphate pH 6.5 and immobilized on multiwell slides coated with polylysine. Slides were immersed in ice-cold methanol (6 min) followed by ice-cold acetone (30 s). Slides were dried, washed once in PBS, and wells were incubated with 1μg/ml DAPI (4',6-diamidino-2-phenylindole) for 1 min. Slides mounted in Vectashield (Vector Laboratories, Burlingame, CA) were sealed with rubber cement or nail polish. Samples were analyzed with a Nikon Optiphot at 1,000 X magnification. Cells highly overexpressing GFP or one of its fusion proteins were excluded from further analysis to avoid the nonlinear range of fluorescence signals. For each set of experiments, images were exposed for the same time, and negatives were processed with Photoshop 5.5.

Nuclear import of cNLS-containing proteins

Classical nuclear transport was analyzed essentially as described (3). The reporter protein NLS-GFP carries the simple SV40-NLS; inhibition of classical nuclear import increases levels of NLS-GFP in the cytoplasm (12).

Isolation of protein complexes containing His_6-Nmd5p and Ssa4p(1-236)-GFP

Yeast cells were grown at room temperature in drop-out medium containing 2% galactose to an OD_{600} of 1.0 to 1.3. Treatment with 10% ethanol was for 10 min at room temperature. Cells were collected by centrifugation, washed once in distilled water and stored at −70°C. Yeast cells equivalent to 10 OD_{600} units were lysed with glass beads in buffer A (20 mM Pipes/KOH, 250 mM sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate, pH 6.8, 1% Triton X-100, 0.5 mM DTT, 1 mM PMSF, 5 mM benzamidine, and protease inhibitors [antipain, aprotinin, chymostatin, leupeptin and pepstatin; each at 1 μg/ml]). Supernatants obtained after 2 min centrifugation (microfuge, 15,000 rpm, 4°C) were incubated with Ni-NTA agarose (Qiagen, Hilden, Germany) with gentle agitation for 2 h at 4°C. The resin was collected by centrifugation and washed three times with 5 mM imidazole in buffer A without Triton X-100 and DTT. For Western blot analysis bound material was eluted by boiling for 10 min in gel sample buffer.

Purification of GST-Gsp1pQ71L and isolation of His_6-Nmd5p/ Ssa4p(1-236)-GFP protein complexes

GST-Gsp1pQ71L (plasmid kindly provided by G. Schlenstedt) was purified from *E. coli* using standard procedures. For affinity purification of Nmd5p/Ssa4p complexes 5 μM GST-Gsp1pQ71L was added together with 1 mM GTP before the purification of protein complexes by metal affinity chromatography.

RESULTS

The cytoplasmic hsp70 Ssa4p translocates rapidly into nuclei upon exposure to ethanol

In the yeast *S. cerevisiae* as in other eukaryotes, cytoplasmic hsp70s accumulate in nuclei in response to various stresses. To follow the distribution of the hsp70 Ssa4p, we have fused *Aequorea victoria* green fluorescent protein (GFP) to different portions of the heat shock protein. In wild-type cells, 10% ethanol triggered the nuclear accumulation of GFP-Ssa4p(16-642),

which was concentrated in nuclei when analyzed after a 10-min treatment (Fig. 1*A*, panel *d*). Thus, our experiments established that Ssa4p, a member of the cytoplasmic *SSA*-protein family, accumulates in nuclei after a short incubation with ethanol.

The N-terminal domain of Ssa4p promotes nuclear accumulation in stressed cells

Having shown that Ssa4p concentrates in nuclei of ethanol-treated cells, we next generated a fusion between the N-terminal 236 amino acid residues of Ssa4p and GFP. This fusion protein, termed Ssa4p(1-236)-GFP, does not contain the bipartite cNLS present in Ssa4p. With a molecular mass greater than 50 kDa, Ssa4p(1-236)-GFP is too large to efficiently diffuse across the nuclear envelope (33). Under normal growth conditions, Ssa4p(1-236)-GFP was cytoplasmic and nuclear in wild-type cells (Fig. 1*B*, panel *b*). The same distribution was obtained under nonstress conditions with several yeast strains, including various mutants (Fig. 5, 6 below and not shown). Like GFP-Ssa4p(16-642), Ssa4p(1-236)-GFP accumulated in nuclei upon 10 min exposure to ethanol (Fig. 1*B*, panel *d*). For comparison of Ssa4p(1-236)-GFP trafficking with classical import, the nuclear reporter protein NLS-GFP, which carries the simple SV40-NLS fused to GFP, was also located. Classical nuclear import was inhibited efficiently by treating cells with ethanol. As a consequence, increased levels of NLS-GFP, which is normally concentrated in nuclei (Fig. 1*C*, panel *b*), were detected in the cytoplasm (Fig. 1*C*, panel *d*). This inhibition of classical nuclear import in response to ethanol was also seen for mutant strains used in our studies, demonstrating that the various mutations in nuclear transport factors do not obstruct the NPC in response to stress exposure (Fig. 2, and data not shown). In contrast to GFP-Ssa4p(16-642), Ssa4p(1-236)-GFP, and NLS-GFP, the GFP-tag was not affected by stress; this protein was nuclear as well as cytoplasmic under all conditions (not shown).

Other forms of insults, such as osmotic or oxidative stress, have been shown to relocate proteins to the nucleus. For instance, the HOG pathway can be activated by sodium chloride, resulting in rapid Hog1p nuclear accumulation upon mild, but not severe, osmotic stress (39–41). However, neither mild nor severe osmotic stress was effective in concentrating Ssa4p(1-236)-GFP in nuclei (Fig. 1*B*, Table 1). In addition, several different conditions were tested for hydrogen peroxide, an agent that induces the nuclear localization of the transcription factor yAP-1 and inhibits classical nuclear import (11, 42). Like osmotic stress, oxidative stress failed to accumulate Ssa4p(1-236)-GFP or GFP-Ssa4p(16-642) in nuclei (Fig. 1*B*, Table 1 and data not shown).

The Star-sequence does not promote nuclear accumulation in ethanol-treated cells

We have previously identified a short hydrophobic segment of Ssa4p, comprising amino acid residues 162 to 171, as a signal that is sufficient to locate the cytoplasmic protein β-galactosidase to nuclei of starving cells (33). This peptide, termed Star (for starvation), was tested for its capacity to accumulate a Star-β-galactosidase fusion protein in nuclei. However, ethanol treatment did not stimulate nuclear import of Star-β-galactosidase in wild-type cells (Fig. 3).

Nuclear accumulation of Ssa4p(1-236)-GFP in response to ethanol is reversible

To determine whether ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP is reversible, cells were treated with 10% ethanol for 10 min and transferred for stress recovery to ethanol-free medium containing glucose, which represses de novo transcription of the *SSA4(1-236)-GFP* gene. Upon 30 min of incubation, Ssa4p(1-236)-GFP nuclear accumulation was reduced, and 2 h

after transfer to the fresh medium the protein had equilibrated between nucleus and cytoplasm (Fig. 4*A*, *B*). To exclude the possibility that Ssa4p(1-236)-GFP in the cytoplasm represents protein translated from pre-existing mRNA, the experiment was repeated in the presence of cycloheximide. The protein synthesis inhibitor was present throughout the stress and recovery period (Fig. 4*C*). Although we detected a larger percentage of cells with weak nuclear accumulation (N≥C) after 1 h recovery, results obtained for 2 h recovery were similar to those of samples incubated without the inhibitor. Thus, the concentration of Ssa4p(1-236)-GFP in nuclei of stressed cells is a reversible process, suggesting that the protein shuttles between nucleus and cytoplasm.

Nuclear accumulation of Ssa4p is independent of Srp1p but requires Gsp1p, Rna1p, and Prp20p

Several soluble factors are required for classical nuclear transport, and mutations in *SRP1*, *GSP1*, *RNA1* and *PRP20* are known to prevent cNLS-dependent nuclear import under nonpermissive conditions (43–45; reviewed in 21). To test the role of these components in nuclear transport of the N-terminal domain of Ssa4p, we incubated conditionally lethal mutants at the nonpermissive temperature to inactivate the mutant transport factors. These pretreated cells failed to import cNLS-containing substrates (43–45 and data not shown).

Following incubation at the nonpermissive temperature, cells were treated with ethanol (Fig. 5). As described above, 10 min exposure to ethanol accumulated Ssa4p(1-236)-GFP in nuclei of wild-type cells. This was observed for all of the parental wild-type strains used in this study (Fig. 5*A–D*, panel *d*). Likewise, after preincubation at the nonpermissive temperature, cells carrying the *srp1-31* allele concentrated Ssa4p(1-236)-GFP in nuclei in response to ethanol (Fig. 5*A*, panel *h*). Thus, Srp1p, the α-subunit of the cNLS-receptor, is not essential for stress-mediated nuclear import of Ssa4p(1-236)-GFP in response to ethanol. These data are consistent with the idea that nuclear import of the N-terminal domain of Ssa4p is not mediated by the classical import route, but by a specialized pathway.

The same strategy as described for Srp1p was employed to determine whether Gsp1p, Rna1p, or Prp20p are involved in nuclear import of Ssa4p(1-236)-GFP (Fig. 5*B–D*). After pretreatment of cells at 37°C and subsequent exposure to ethanol, parental wild-type cells accumulated Ssa4p(1-236)-GFP in nuclei (Fig. 5*B–D*, panel *d*). In contrast, Ssa4(1-236)-GFP failed to concentrate in nuclei of *gsp1-2*, *rna1-1* or *prp20-1* cells (Fig. 5*B–D*, panel *h*), demonstrating that Gsp1p, Rna1p, and Prp20p are required for stress-dependent nuclear import of the N-terminal domain of Ssa4p.

Nuclear accumulation of Ssa4p upon ethanol exposure requires the carrier Nmd5p

Members of the importin-β family are involved in nucleocytoplasmic trafficking of a variety of cargos (reviewed in 46). Moreover, nuclear transport mediated by these carriers depends on the small GTPase Gsp1p and its interacting factors. To test the potential role of importin-β proteins in Ssa4p(1-236)-GFP nuclear transport wild-type and mutant cells carrying a knockout in one of the importin-β genes were incubated with ethanol for different periods of time (Fig. 6*A–C*). This strategy identified Nmd5p as the carrier necessary for the rapid nuclear accumulation of Ssa4p(1-236)-GFP in response to ethanol treatment. In contrast to the inactivation of *NMD5*, mutations in several other importin-β family members did not prevent Ssa4p(1-236)-GFP from concentrating

in the nucleus (Fig. 6*A*, *C*). Like the N-terminal portion of Ssa4p, GFP-Ssa4p(16-642) also required the carrier Nmd5p to accumulate in nuclei of cells that had been exposed to ethanol (Fig. 6*B*). In further experiments, we determined the kinetics of Ssa4p(1-236)-GFP nuclear concentration. All of the mutant strains, except *nmd5::TRP1*, accumulated Ssa4p(1-236)-GFP in nuclei after 2 min incubation with 10% ethanol (Fig. 6*C*). In the presence of ethanol, this nuclear accumulation persisted for at least 60 min.

Although deficient in nuclear import of Ssa4p upon ethanol treatment, *nmd5::TRP1* cells were not defective in classical nuclear import (Fig. 2). NLS-GFP accumulated in nuclei of control cells and relocated to the cytoplasm upon exposure to ethanol. As expected, in control experiments, *nmd5::TRP1* cells were unable to accumulate Hog1p-GFP in nuclei in response to osmotic stress (Fig. 7).

Effect of ethanol stress on the viability of yeast cells

We further characterized the toxicity of ethanol by monitoring the viability of wild-type and *nmd5::TRP1* strains that had been treated with alcohol. After exposure to 10% ethanol for 10 min, cells were immediately diluted and plated out. The number of colonies formed was compared with untreated control cells. For wild-type and mutant cells synthesizing Ssa4p(1-236)-GFP, the number of colonies was reduced to 60–70% of the unstressed controls (Fig. 8). Similar results were obtained for untransformed cells, in line with the idea that the majority of cells recover from ethanol-induced damage (Fig. 8, and data not shown).

Nmd5p and Ssa4p interact in growing yeast cells

Because *nmd5::TRP1* cells failed to accumulate Ssa4p(1-236)-GFP in nuclei in response to ethanol treatment, it was conceivable that Nmd5p and Ssa4p can associate to form import complexes. To analyze such a potential interaction between Nmd5p and Ssa4p(1-236)-GFP, His_6-tagged Nmd5p and Ssa4p(1-236)-GFP were simultaneously synthesized in yeast. Metal affinity chromatography was used to purify protein complexes that contained His_6-Nmd5p and Ssa4p(1-236)-GFP (Fig. 9*A*, part *a*). Under these conditions, similar amounts of His_6-Nmd5p were purified from control and stressed cells (Fig. 9*A*, parts *c* and *f*). The interaction between His_6-Nmd5p and Ssa4p(1-236)-GFP was not only observed when both proteins were synthesized in the same yeast cell, both proteins also copurified when yeast extracts containing either protein were mixed before affinity purification (Fig. 9*A*, part *b*). Control experiments demonstrated the specificity of this interaction, as Ssa4p(1-236)-GFP did not bind to the resin in the absence of His_6-Nmd5p (Fig. 9*A*, part *a*).

Nuclear import complexes containing a member of the importin-β family of carriers can be dissociated by the GTP-bound form of Ran/Gsp1p (reviewed in 19). When purified GST-Gsp1p-Q71L, a mutant that mimics the GTP-bound form of Gsp1p, was added to the binding assay, the purification of His_6-Nmd5p was not affected (Fig. 9*A*, part *e*). When the amounts of His_6-Nmd5p purified under different conditions were compared, a maximum variation of 3 ± 3.7% was observed (Fig. 9*A*, part *f*). By contrast, the levels of Ssa4p(1-236)-GFP that copurified with His_6-Nmd5p were drastically reduced by GST-Gsp1pQ71L (Fig. 9*A*, part *d*, *f*; ** $P<0.01$). This effect of GST-Gsp1pQ71L on the formation of His_6-Nmd5p/Ssa4p(1-236)-GFP complexes was detected for unstressed as well as ethanol-treated cells. As shown in Fig. 9, exposure to ethanol was not a prerequisite for the association between His_6-Nmd5p and Ssa4p(1-236)-GFP.

However, the amount of Ssa4p(1-236)-GFP complexed with His_6-Nmd5p was increased significantly by incubation with ethanol (Fig. 9*A*, part *f*; x, P=0.04).

In most of our experiments anti-GFP antibodies recognized two major bands in cells synthesizing Ssa4p(1-236)-GFP, but the faster migrating protein did not associate with His_6-Nmd5p. This faster migrating band could be a proteolytic product of Ssa4p(1-236)-GFP. Alternatively, the higher molecular mass form might have been modified post-translationally. At present, we cannot distinguish between these possibilities.

The specificity of the Nmd5p/Ssa4p(1-236)-GFP interaction was further analyzed in additional control experiments, showing that the GFP-tag did not associate with His_6-Nmd5p (Fig. 9*B*). When cells were cotransformed with plasmids encoding His_6-Nmd5p and GFP, only low-level synthesis of GFP was obtained (not shown). Therefore, crude cell extracts containing either His_6-Nmd5p or GFP were mixed before affinity chromatography. GFP did not interact with Nmd5p (Fig. 9*B*, part *b*), whereas Ssa4p(1-236)-GFP bound to the carrier under these conditions (Fig. 9*A*, part *b*).

Ethanol stimulates the association of Nmd5p-GFP with NPCs

Results described in the previous section demonstrate that the association between Nmd5p and Ssa4p(1-236)-GFP was stimulated by the incubation with ethanol. To test whether a subsequent step of nuclear import, i.e., binding of Nmd5p to NPCs, is regulated by ethanol, we have used two different NPC clustering strains, *rat2-1* and *nup133Δ*. Both strains form NPC clusters when incubated at room temperature, but mRNA export and classical nuclear import are not affected (22 and references therein). We located NPC clusters in these cells by staining with mab414, an antibody that binds to FXF-containing nucleoporins (Fig. 10*A*, *B*). Binding of Nmd5p-GFP to clusters was then quantified for different experimental conditions. In untreated controls, about one-third of the cells displayed association of Nmd5p-GFP with NPC clusters (Fig. 10*B*). In response to ethanol, however, the interaction of Nmd5p-GFP with NPC clusters was significantly increased in both strains. This enhanced binding of Nmd5p-GFP was observed after 2 min exposure to ethanol, and similar results were obtained after 10 min incubation. These results are comparable with the kinetics of Ssa4p(1-236)-GFP nuclear accumulation in various strains carrying the *NMD5* wild-type allele, which concentrated most of Ssa4p(1-236)-GFP in nuclei after 2 min exposure to ethanol (Fig. 6*C*). In control experiments, binding of Nmd5p-GFP to NPCs was tested upon osmotic stress. As expected (25), 10 min incubation with 0.4 M NaCl significantly increased the association of Nmd5p with NPC clusters (Fig. 10*B*).

Stress-induced nuclear accumulation of Ssa4p(1-236)-GFP relies on Pkc1p

Given the effect of ethanol on membrane fluidity and the important role of Pkc1p, Mid2p, and Wsc1p in the cell integrity pathway and of Pmt2p in regulating Mid2p sensor activity, it was possible that these components are involved in the targeting of Ssa4p to the nucleus of ethanol-treated cells. We have tested this hypothesis by analyzing the nuclear accumulation of Ssa4p(1-236) in knockout mutants *pkc1Δ*, *wsc1Δ*, *mid2Δ*, *pmt2Δ*, and the double mutant *wsc1Δ mid2Δ*. In all of these mutants, the nuclear accumulation of Ssa1p(1-236)-GFP after ethanol treatment was severely impaired (Fig. 11*A*, *B*). In particular, mutants *pkc1Δ*, *mid2Δ*, and the double mutant *wsc1Δ mid2Δ* failed to respond properly to ethanol stress, whereas a minor nuclear accumulation of Ssa4p(1-236)-GFP was observed for *wsc1Δ* (Fig. 11*B*). Unlike these mutants, parental wild-

type strains did not show a defect in the ethanol-induced nuclear accumulation of the reporter protein (Fig. 11 and data not shown).

DISCUSSION

Ssa4p, a member of the cytoplasmic hsp70 family, concentrates in the nucleus when cells are exposed to ethanol. By contrast, other types of insults, such as osmotic or oxidative stress, fail to induce the nuclear accumulation of Ssa4p. These results suggest that ethanol produces a specific cellular response for which Ssa4p is a downstream target.

With the experiments described here, we have demonstrated that the N-terminal 236 amino acid residues of Ssa4p are sufficient to mediate the nuclear accumulation of the non-nuclear passenger GFP when cells are exposed to ethanol. This N-terminal portion of Ssa4p does not contain a cNLS, consistent with the idea that a nonclassical signal mediates stress-induced nuclear import. In line with this hypothesis, ethanol-induced nuclear import of Ssa4p does not depend on Srp1p, the α-subunit of the cNLS-receptor, but requires the importin-β Nmd5p. Although Ssa4p nuclear import caused by ethanol has unique requirements, it shares components with the classical import machinery. In particular, the Gsp1p-GTPase system plays a role in nuclear import, as well as nuclear export of a variety of substrates. Our analyses of conditionally lethal mutants reveal that Gsp1p and its modulating factors Rna1p and Prp20p are necessary to accumulate Ssa4p(1-236)-GFP in nuclei when cells have been treated with ethanol.

A hallmark of stress-dependent inhibition of classical nuclear import is the collapse of the Gsp1p concentration gradient. Under normal growth conditions, Gsp1p is predominantly nuclear, whereas ethanol stress dissipates the Gsp1p concentration gradient across the nuclear envelope (12). However, cells exposed to ethanol still carry out the Gsp1p GTPase cycle (10). Differences in the requirement for the mammalian Gsp1p homologue Ran with respect to various nuclear transport pathways have been reported (47–51; reviewed in 20). As such, importin-β can be translocated through NPCs in a Ran-independent fashion (48, 49). On the basis of these observations, we propose that the stress-induced nuclear accumulation of cytoplasmic hsp70s requires Gsp1p, but not a steep concentration gradient of the GTPase. It is likely that the amount of Gsp1p-GTP left in the nucleus is sufficient to support Ssa4p(1-236)-GFP nuclear import in ethanol-treated cells.

The complete GTPase cycle, with both Gsp1p in its GTP-bound and GDP-bound form, is necessary for Ssa4p(1-236)-GFP nuclear transport. These results are in agreement with the requirement for the carrier Nmd5p for Ssa4p import into nuclei of ethanol-treated cells. Like other importin-β family members, Nmd5p depends on the Gsp1p GTPase cycle to translocate cargo across the nuclear pore complex. We have now identified the hsp70 Ssa4p as a novel transport substrate for Nmd5p whose nuclear import is induced by ethanol exposure. Furthermore, our experiments show that the N-terminal part of Ssa4p is sufficient to promote the specific association with Nmd5p in growing cells. This association can be disrupted by Gsp1p-GTP, demonstrating that the interaction Nmd5p/Ssa4p(1-236)-GFP has the properties of a bona fide import complex.

While Nmd5p and Ssa4p(1-236)-GFP can associate in unstressed cells, ethanol treatment stimulates significantly the binding of Ssa4p(1-236)-GFP to Nmd5p. This increase in import complexes is likely to contribute to the nuclear accumulation of Ssa4p(1-236)-GFP. In addition

to the stimulation of import complex formation, subsequent steps in Ssa4p(1-236)-GFP nuclear transport may also be affected by ethanol. Indeed, we found the interaction of Nmd5p with NPCs to be up-regulated significantly by exposure to ethanol. Taken together, our results suggest that the increase in import complex generation as well as the enhanced binding of Nmd5p to NPCs stimulate the nuclear accumulation of Ssa4p(1-236)-GFP.

As various alcohols affect the fluidity of membranes and potentially also the diffusion limit of the NPC (52), it might be assumed that leakage across the nuclear envelope plays a role in the relocation of proteins between nucleus and cytoplasm. As such, Ssa4p(1-236)-GFP may traverse the NPC by diffusion and accumulate in nuclei because of retention. However, several lines of evidence argue against such a simple diffusion/retention mechanism. For instance, the use of ethanol in wild-type cells failed to increase the diffusion limit of NPCs (52). More importantly, diffusion/retention is unlikely to depend on the Gsp1p-GTPase system and the carrier Nmd5p, components which we have shown to be required for the ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP. Although we do not rule out the possibility that retention contributes to the nuclear concentration of Ssa4p(1-236)-GFP after its import, our results clearly demonstrate that nuclear accumulation requires a nonclassical nuclear transport pathway.

One potential complication of the experiments discussed here is that the expression levels of genes encoding GFP-fusions might influence the localization of reporter proteins. Such a process would affect, in particular, proteins of low abundance, and it might result in their leakage across the nuclear envelope. However, in the case of cytoplasmic hsp70s, even normal growth conditions require that at least one member of the Ssa protein family is present at high concentrations to ensure cell viability (7 and references therein). Furthermore, as discussed above, Ssa4p(1-236)-GFP relocation via a leakage/nuclear retention mechanism is unlikely, as the Ssa4p(1-236)-GFP nuclear accumulation depends on a nonclassical transport route. In the case of NLS-GFP, the reporter protein for classical nuclear import, previous studies have shown that under the conditions used in our experiments NLS-GFP is a reliable tool to monitor nuclear transport in growing yeast cells (3, 12, 18, 33 and references therein).

Although cells that do not synthesize Nmd5p fail to import Ssa4p into nuclei of ethanol-treated cells, inactivation of the *NMD5* gene does not reduce the viability of cells upon ethanol exposure. One possible explanation for this result could be that ethanol-induced damage triggers a variety of repair processes, one of which is Ssa4p targeting to nuclei. However, it is feasible that other responses are also necessary to recover from stress and restore the normal cell physiology. As such, it is conceivable that ethanol will affect the fluidity of membranes and the proper function of membrane-associated proteins. If this is the case, nuclear transport of Ssa4p and the presence of Nmd5p may not be the limiting factors that regulate the survival of ethanol-stressed cells.

As discussed above, our experiments clearly show that the cNLS of Ssa4p is not required for ethanol-induced nuclear accumulation. We have further attempted to define the minimal region within the N-terminal domain of Ssa4p that is sufficient for nuclear localization in stressed cells. However, smaller segments of Ssa4p were not stable when synthesized in yeast, which prevented the analysis of their potential targeting function (not shown). Nevertheless, we have tested whether the Star-sequence of Ssa4p, which can direct β-galactosidase to the nucleus upon starvation (33), mediates nuclear accumulation in ethanol-treated cells. Unlike Ssa4p(1-236)-GFP, Star-β-galactosidase failed to concentrate in nuclei of cells treated with ethanol. These

results suggest that the N-terminal domain of Ssa4p provides more than one nuclear import signal for nuclear accumulation in response to distinct types of stress. The presence of multiple import signals with different transport characteristics has been reported previously for ribosomal proteins (53). The use of divergent transport sequences after exposure to different environmental challenges may offer the advantage to control the stress response and fine-tune the nucleocytoplasmic distribution of Ssa4p, according to the physiological requirements of the cell.

Our data demonstrate that ethanol, but not other environmental stresses like oxidants or osmotic stress, promotes nuclear import of Ssa4p. This indicates that a specific response is triggered in cells that have been treated with ethanol, distinct from the signaling events activated upon treatment with hydrogen peroxide or salt. Indeed, we have shown here that Ssa4p(1-236)-GFP fails to accumulate in nuclei of mutants that are lacking Pkc1p or one of the major sensors of cell integrity, that is, Wsc1p and Mid2p. Both Wsc1p and Mid2p are proteins of the cytoplasmic membrane where they act as upstream regulatory sensors that can activate Pkc1p (reviewed in 25). Wsc1p and Mid2p have been proposed to have partially overlapping functions, with Wsc1p playing a major role in vegetative growth, whereas Mid2p is mostly involved in mating (25, 30, 31). Our results show that Wsc1p and Mid2p are both required for Ssa4p(1-236)-GFP nuclear accumulation in ethanol-stressed cells during vegetative growth. Mid2p seems to play a more important role in this process, as *mid2Δ* mutants were more severely affected in nuclear import of the reporter protein. Like *mid2Δ*, the mutant *pkc1Δ* failed to respond properly to ethanol stress and did not concentrate Ssa4p(1-236)-GFP in nuclei. On the basis of these results, we propose that Mid2p and Wsc1p sense ethanol-induced changes in the plasma membrane, such as alterations of the bilayer fluidity. This signal is then transmitted to Pkc1p, and Ssa4p could be a downstream target of this pathway, which accumulates in nuclei upon activation of the protein kinase. Future experiments will have to define the molecular components that link Pkc1p activation to the concentration of Ssa4p in nuclei.

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Table 1

Localization of Ssa4p(1-236)-GFP upon exposure to different stresses

Conditions	N>C [%]	N≥C [%]	N+C [%]
Control	11.0 ± 3.0	0	89.0 ± 3.0
10% EtOH, 10 min	86.7 ± 3.2	0	13.3 ± 3.2
0.3 mM H_2O_2, 10 min	14.0 ± 15.1	0	86.0 ± 15.1
0.3 mM H_2O_2, 60 min	1.7 ± 1.5	1.0 ±1.7	97.3 ± 0.6
0.3 mM H_2O_2, 120 min	2.0 ± 2.4	1.0 ± 1.7	97.0 ± 3.0
2 mM H_2O_2, 10 min	12.7 ± 12.1	0	87.3 ± 12.1
0.4 M NaCl, 1 min	0	3.7 ± 2.5	96.3 ± 2.5
0.4 M NaCl, 10 min	7.6 ± 6.7	2.7 ± 4.6	89.7 ± 2.1
1.4 M NaCl, 45 min	5.0 ± 3.0	0	95.0 ± 3.0
1.4 M NaCl, 60 min	1.7 ± 1.5	0	98.3 ± 1.5
1.4 M NaCl, 120 min	0.3 ± 0.6	0	99.7 ± 0.6

Localization of Ssa4p(1-236)-GFP upon exposure to different stresses. The reporter protein was located by fluorescence microscopy in wild-type cells. At least 100 cells expressing the reporter gene were monitored in each experiment. Numbers represent means and S.D. of three independent experiments.

Fig. 1

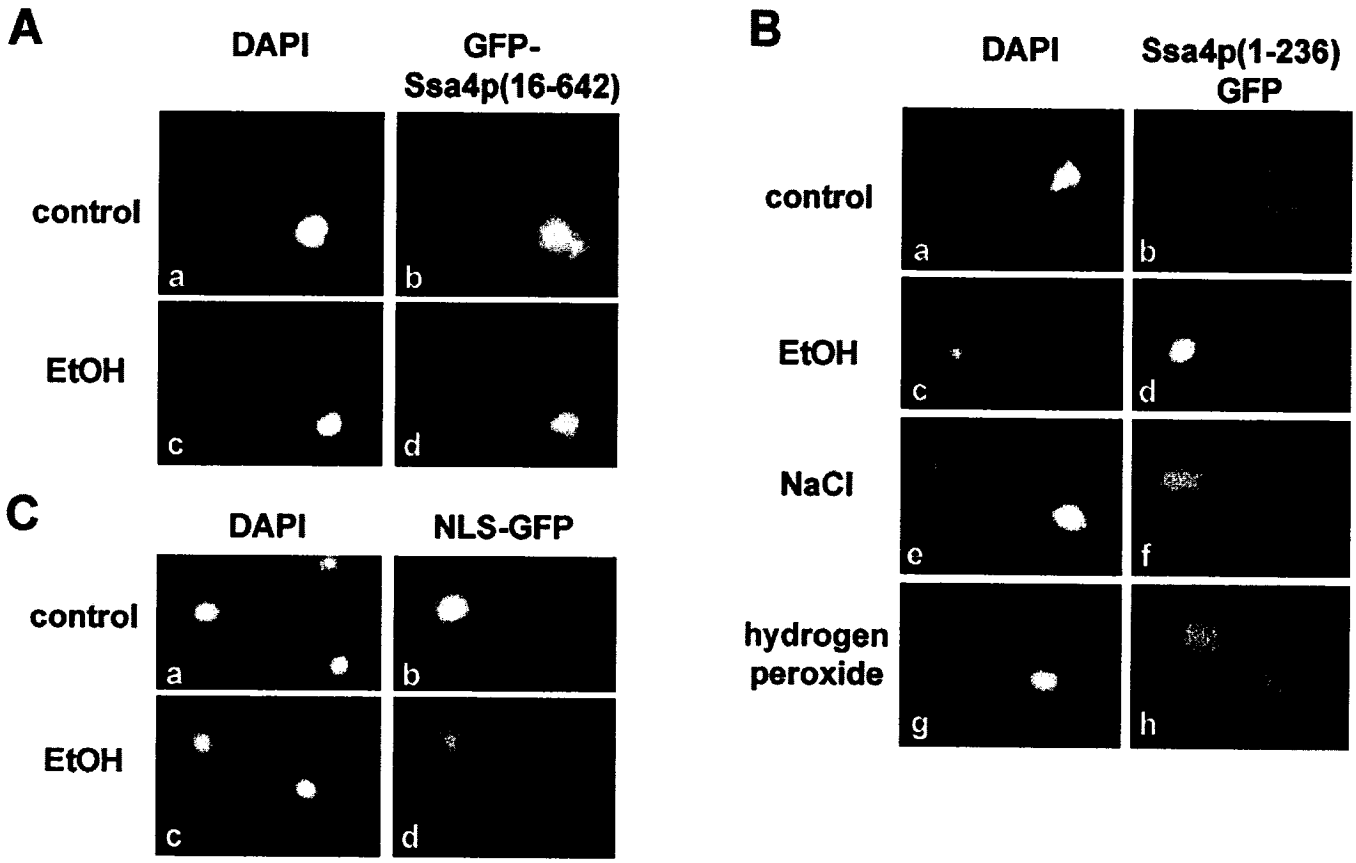


Figure 1. Stress-induced relocation of the cytoplasmic hsp70 Ssa4p and NLS-GFP. Wild-type yeast cells synthesizing Ssa4p fusion proteins or NLS-GFP were grown overnight in drop out medium containing 2% galactose. In fixed cells DNA was visualized with DAPI, and fusion proteins were localized by fluorescence microscopy. The experiment was carried out with (*A*) GFP-Ssa4p(16-642), (*B*) Ssa4p(1-236)-GFP and (*C*) NLS-GFP. GFP-containing reporter proteins were located in control cells (*b*) or after 10 min exposure to 10% ethanol (*d*), 0.4 M NaCl (*f*), or 0.3 mM hydrogen peroxide (*h*).

Fig. 2

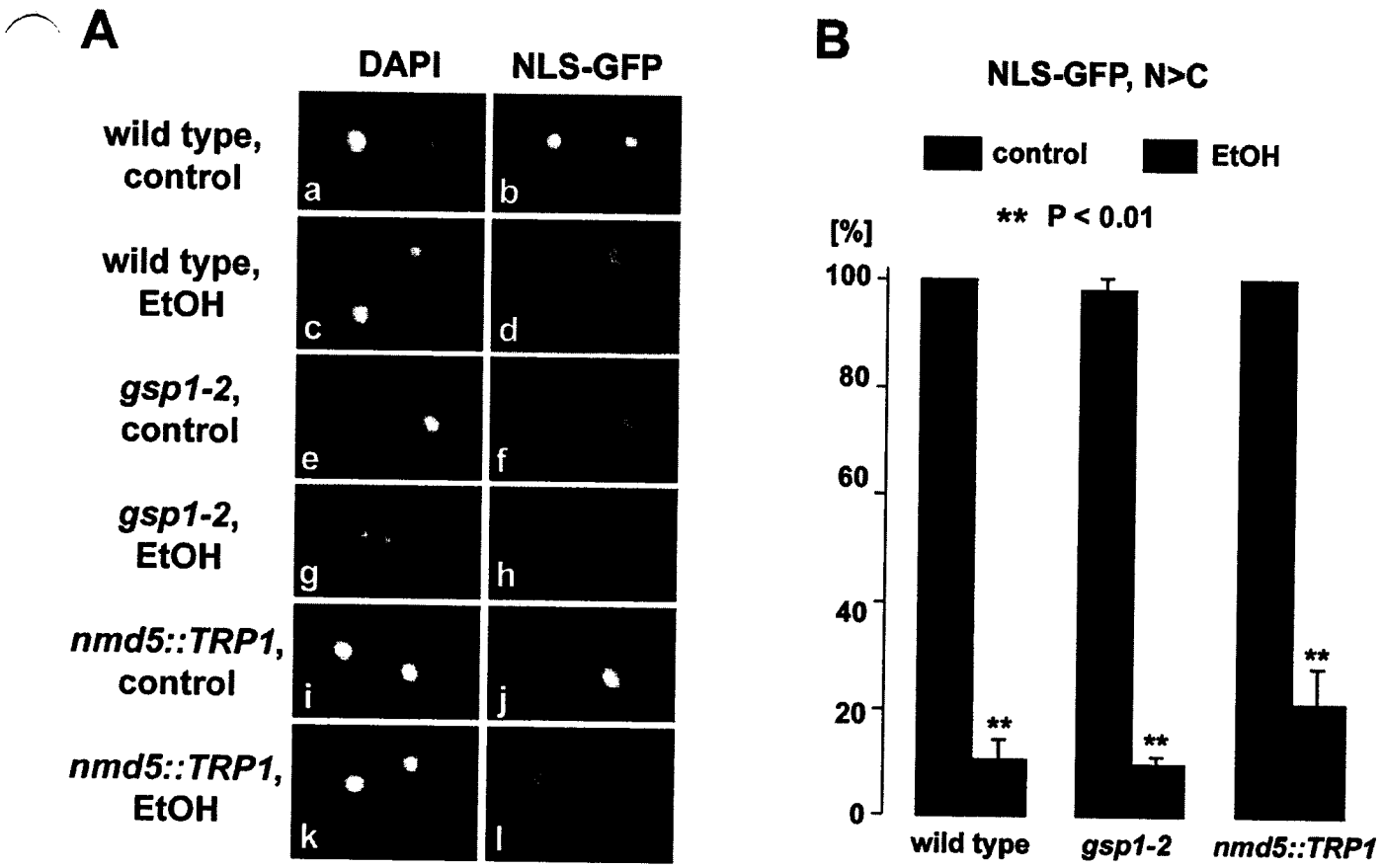


Figure 2. Inhibition of classical nuclear import in wild-type and mutant yeast strains treated with ethanol. (*A*) NLS-GFP was localized by fluorescence microscopy in wild-type cells (***a-d***), *gsp1-2* (***e-h***) and *nmd5::TRP1* (***i-l***) for nonstress conditions (***b, f, j***) and upon 10 min incubation with 10% ethanol (***d, h, l***). After fixation of the cells, DNA was stained with DAPI, and NLS-GFP was located by fluorescence microscopy. (***B***) At least 65 cells were evaluated in three independent experiments for control and stress conditions (EtOH). Means and S.D. are shown.

Fig. 3

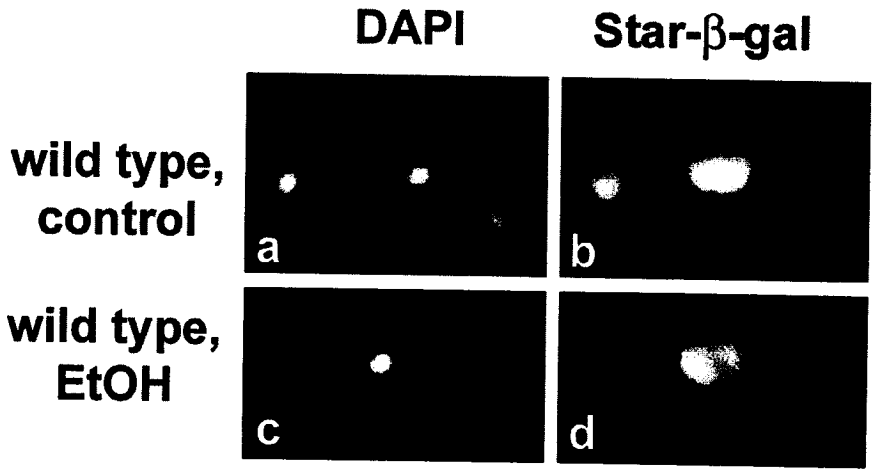


Figure 3. The Star-sequence does not promote nuclear accumulation in ethanol-treated cells. Wild-type cells synthesizing Star-β-galactosidase (Star-β-gal) were tested for the localization of the reporter protein before (*b*) and after (*d*) 10 min incubation with 10% ethanol. Nuclei were visualized with DAPI and Star-β-galactosidase was detected by indirect immunofluorescence with antibodies against β-galactosidase (33).

Fig. 4

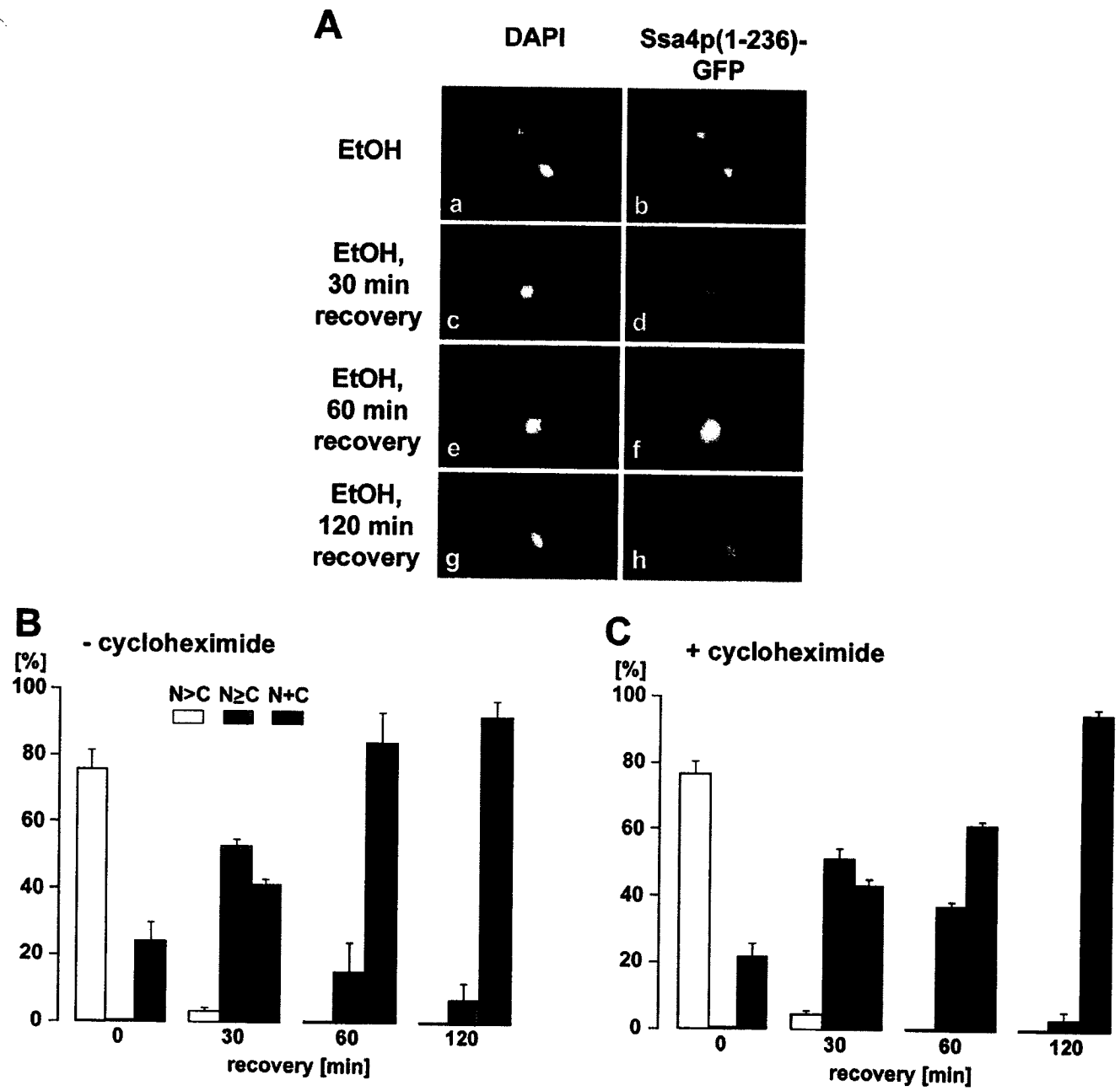


Figure 4. Ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP is reversible. Wild-type cells synthesizing Ssa4p(1-236)-GFP were exposed to 10% ethanol for 10 min at room temperature. Cells were immediately transferred to ethanol free medium containing glucose and incubated at room temperature for the times indicated. (*A*) Fluorescence microscopy of stressed and recovering cells was carried out as described for **Fig. 1**. (*B*) The localization of Ssa4p(1-236)-GFP was determined for different times of recovery. At least 100 cells synthesizing Ssa4p(1-236)-GFP were analyzed in each of three independent experiments. Means and S.D. are shown for the different time points analyzed. (*C*) The experiment shown in part B was carried out in the presence of cycloheximide. The protein synthesis inhibitor was present at a final concentration of 100 µg/ml during the incubation with ethanol and throughout the recovery period. N>C, nuclear accumulation; N≥C, weak nuclear accumulation; N+C, equal fluorescence signals were discovered in nucleus and cytoplasm.

Fig. 5

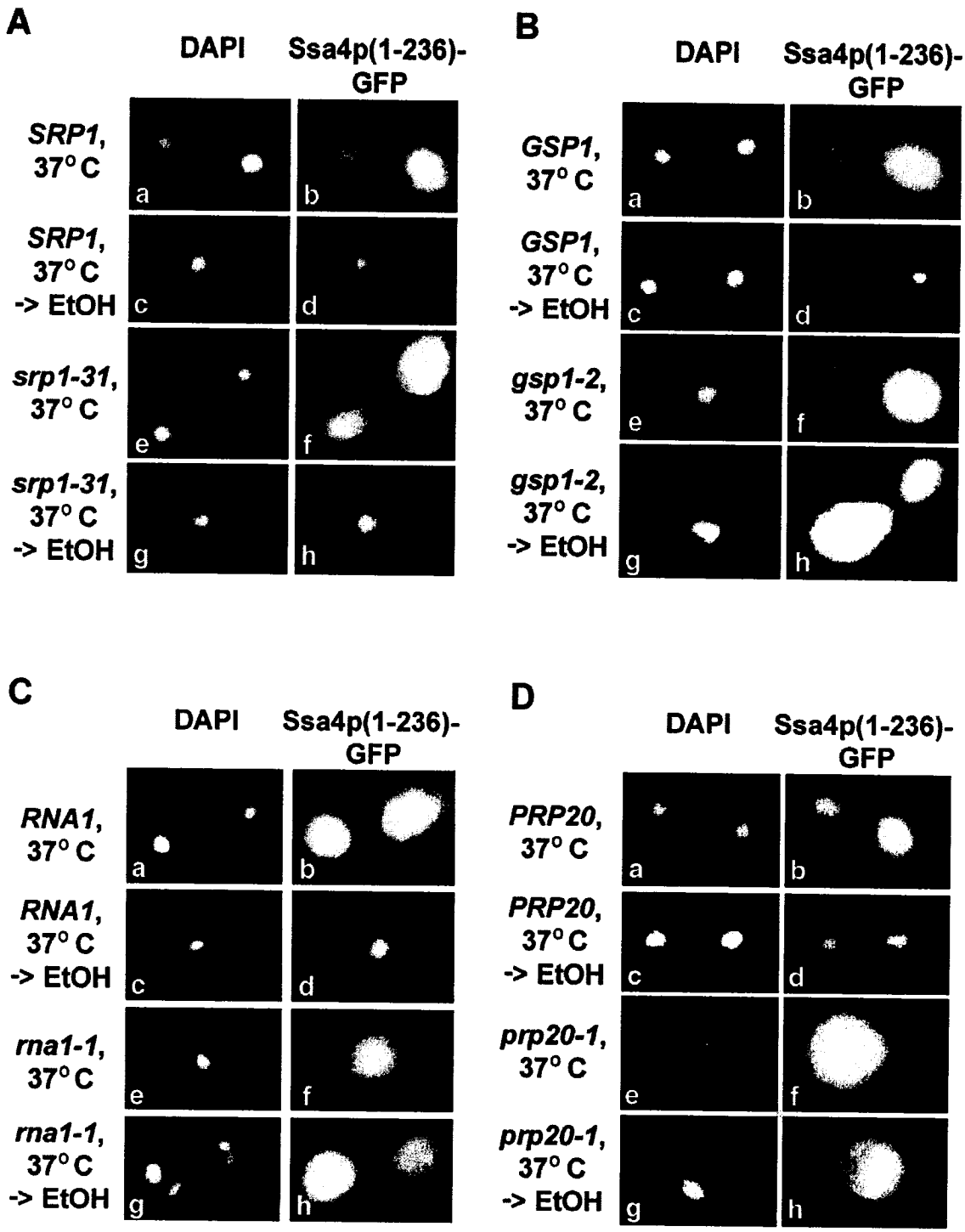


Figure 5. Localization of Ssa4p-GFP in yeast strains carrying conditionally lethal alleles of *SRP1*, *GSP1*, *RNA1*, *PRP20*. Parental wild-type strains and cells mutated in components of the classical nuclear protein import pathway were analyzed for the distribution of Ssa4p(1-236)-GFP under normal and stress conditions. (*A*) *SRP1* (***a-d***) and *srp1-31* (***e-h***) cells were grown at room temperature and incubated for 1 h at 37°C to inactivate srp1-31p. Cells were subsequently incubated for 10 min with 10% ethanol (***c***, ***d***, ***g***, ***h***) and processed as described for **Fig. 1**. Staining of DNA with DAPI and green fluorescence of Ssa4p(1-236)-GFP are shown. ***(B-D)*** Yeast strains carrying the alleles *GSP1*, *gsp1-2*, *RNA1*, *rna1-1*, *PRP20*, or *prp20-1* were analyzed as described for ***(A)***.

Fig. 6

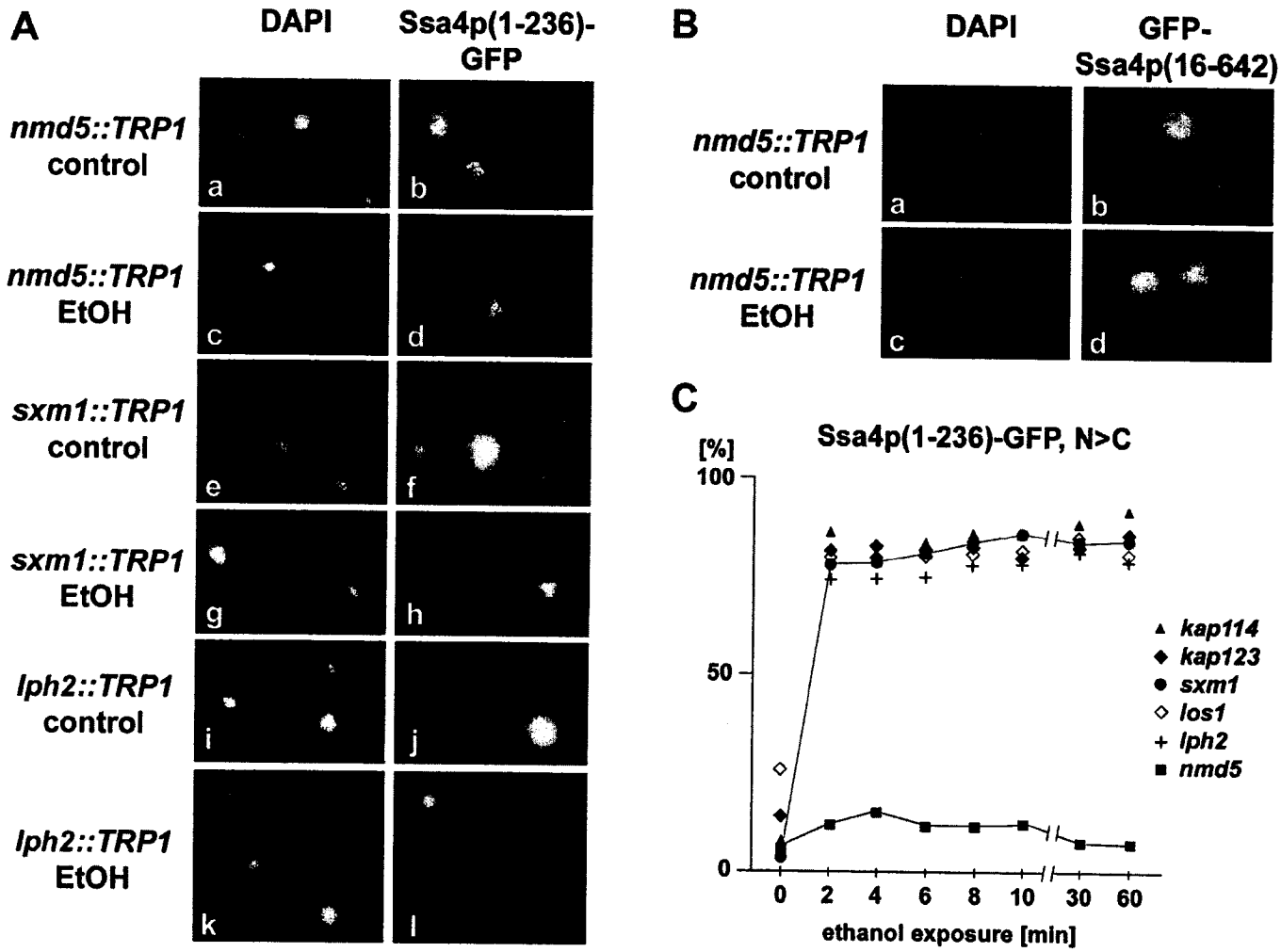


Figure 6. Ethanol-induced nuclear accumulation of Ssa4p(1-236) requires the carrier Nmd5p. (*A*)Yeast strains carrying a mutation in one of the importin-β genes were analyzed for the nuclear accumulation of Ssa4p(1-236) in response to ethanol stress. The distribution of Ssa4p(1-236)-GFP is shown for strains *nmd5::TRP1*, *sxm1::TRP1* and *lph2::TRP1*. Control and ethanol-treated cells were analyzed in parallel. (*B*) GFP-Ssa4p(16-642) was located in *nmd5::TRP1* control and ethanol-stressed cells as indicated. (*C*) Ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP in mutant yeast strains deleted for one of the importin-β genes. Mutant cells synthesizing Ssa4p(1-236)-GFP were stressed with 10% ethanol for the time indicated. Cells were fixed, and nuclear accumulation of the fusion protein was monitored by fluorescence microscopy. Results were observed at least three times. Nuclear accumulation of Ssa4p(1-236)-GFP was determined at the times indicated, 100 cells were scored in each independent experiment for every time point, and means are depicted in the figure.

Fig. 7

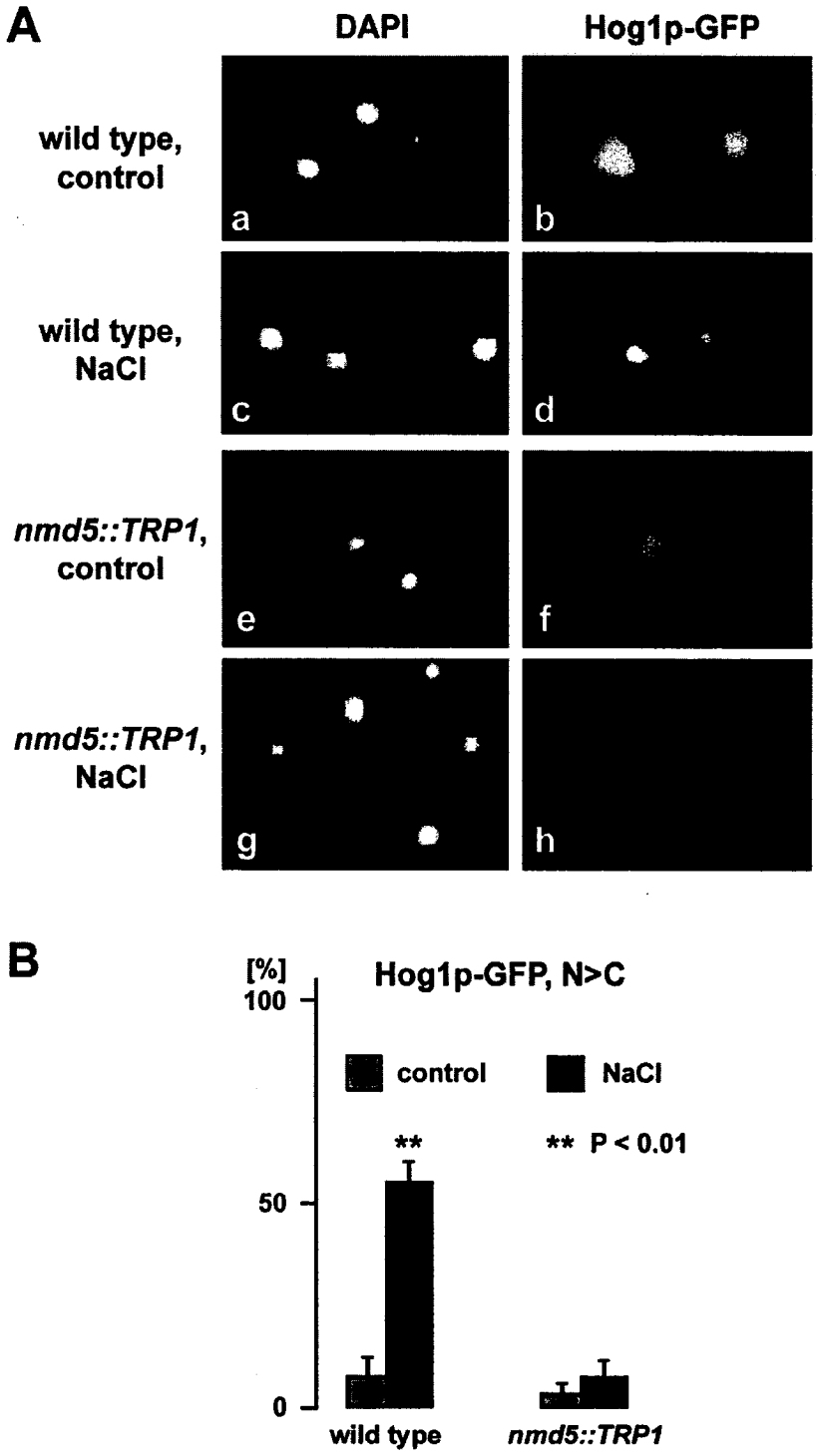


Figure 7. Nuclear accumulation of Hog1p-GFP in wild-type and *nmd5::TRP1* cells. (*A*) Nuclear accumulation of the reporter protein Hog1p-GFP was monitored in unstressed (control) cells and after a 10 min exposure to 0.4 M NaCl. (*B*) The accumulation of Hog1p-GFP (N>C) was monitored in at least 100 cells in each of three separate experiments. Means and S.D. are illustrated for wild-type and *nmd5::TRP1* cells under nonstress and osmotic stress conditions.

Fig. 8

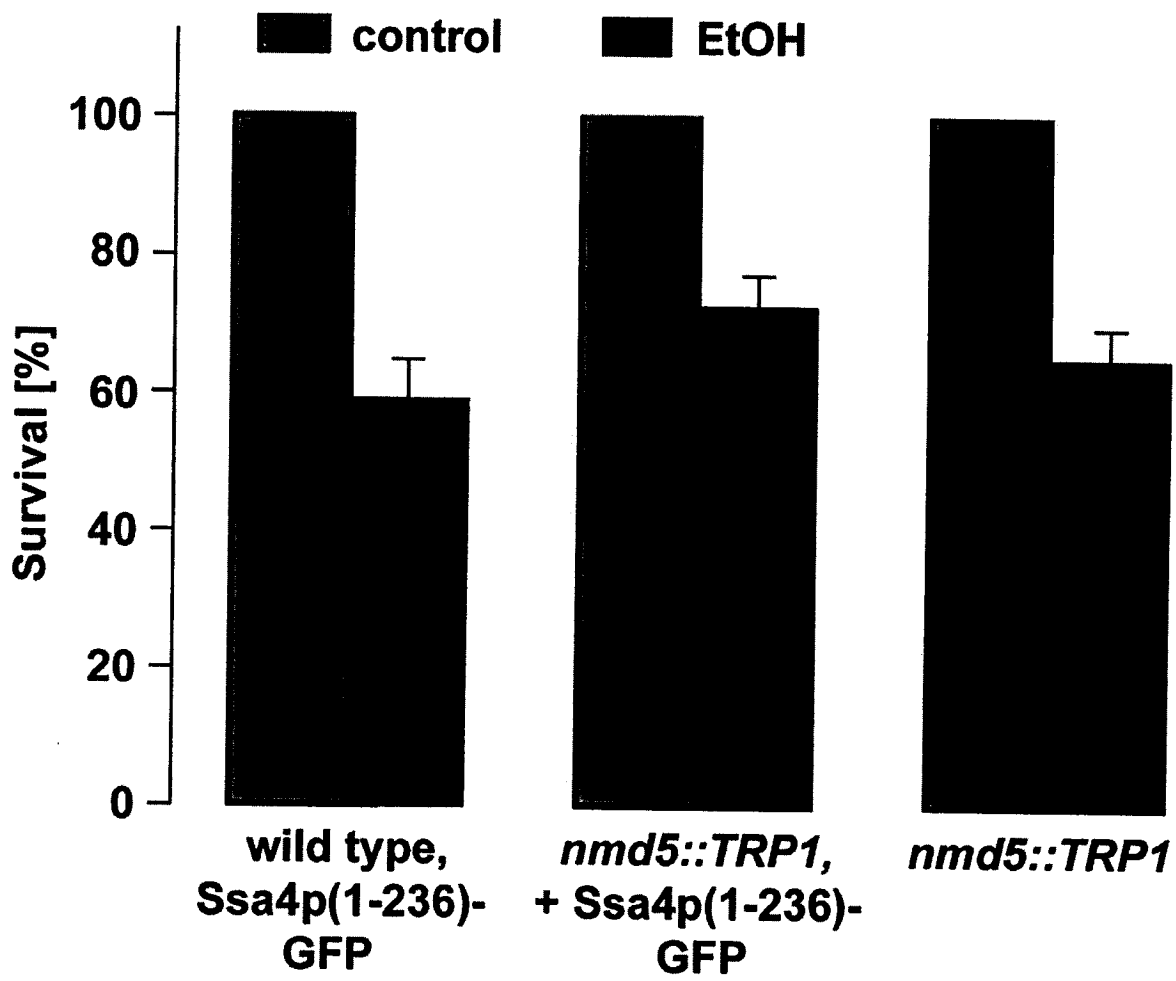
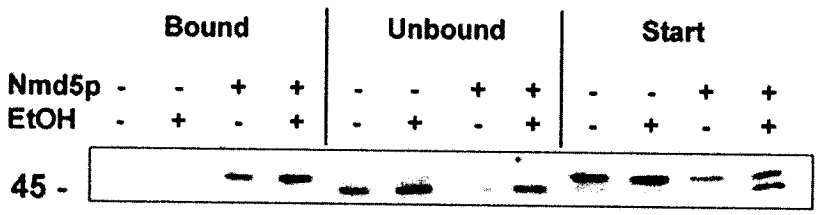


Figure 8. Viability of wild-type and *nmd5::TRP1* cells after ethanol stress. Wild-type and *nmd5::TRP1* cells synthesizing Ssa4p(1-236)-GFP were treated for 10 min with 10% ethanol (EtOH) and immediately plated out. The formation of colonies was compared with unstressed cells (control), which was defined as 100% survival. Results for three independent experiments (means and S.D.) are shown.

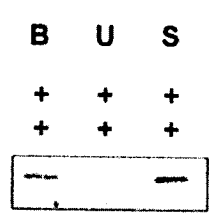
Fig. 9

(A) Ssa4p(1-236)-GFP

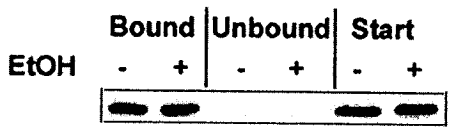
(a) Ssa4p(1-236)-GFP



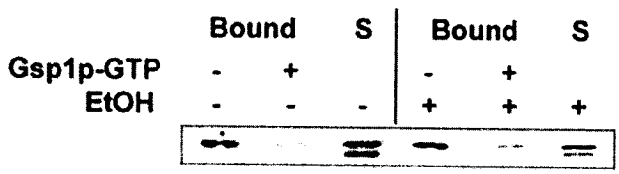
(b)



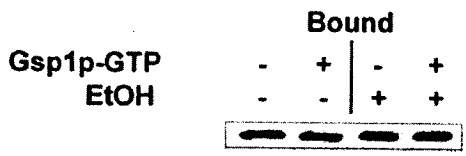
(c) His6-Nmd5p



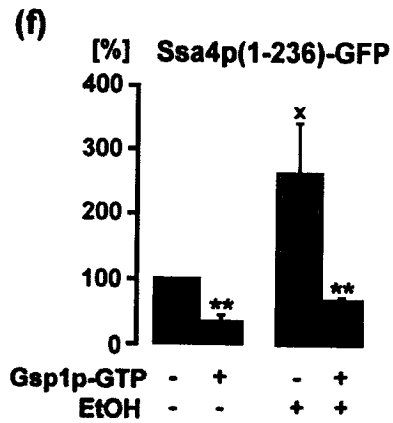
(d) Ssa4p(1-236)-GFP

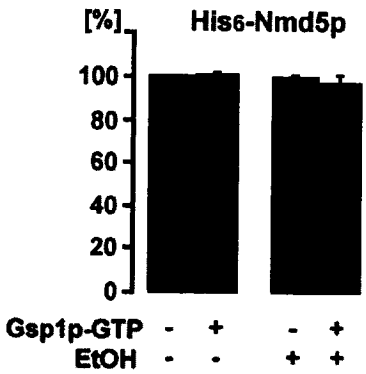


(e) His6-Nmd5p



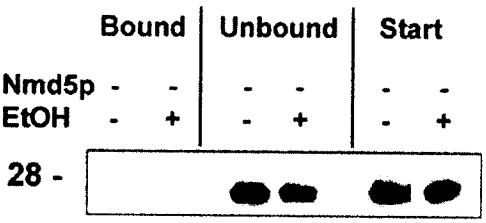
(f)





(B) GFP

(a)



(b)

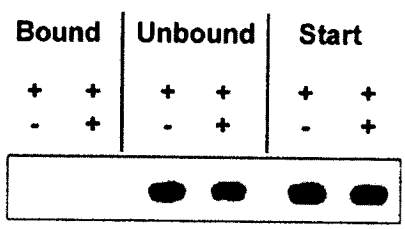
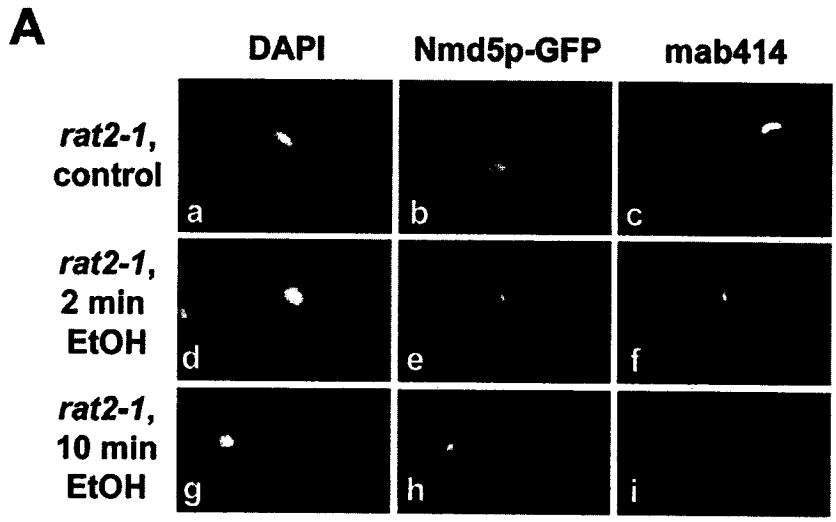


Figure 9. Binding of Ssa4p(1-236)-GFP to His6-tagged Nmd5p. Part (*A*) of the figure shows the copurification of Ssa4p(1-236)-GFP with His_6-Nmd5p, part (*B*) control experiments carried out with the GFP-tag. Material bound to the netal affinity resin (B), 20% of the unbound proteins (U) and 20% of the starting material (S) were analyzed by Western blotting with antibodies against GFP or the His_6-tag. (*a*) Crude extracts were prepared from yeast cells synthesizing His_6-Nmd5p, His_6-Nmd5p and Ssa4p(1-236)-GFP or the GFP-tag as shown in the Figure. (*b*) Extracts were prepared from cells synthesizing His_6-Nmd5p, Ssa4p(1-236)-GFP, or GFP and mixed before affinity purification as indicated. (*c*) The purification of His_6-Nmd5p was monitored under the same conditions as described for (**a**). (*d*) The effect of GST-Gsp1pQ71L (Gsp1p-GTP) on the association of Ssa4p(1-236)-GFP with His_6-Nmd5p was evaluated. Crude extracts from cells synthesizing Ssa4p(1-236)-GFP and His_6-Nmd5p were incubated in the absence or presence of GST-Gsp1pQ71L (Materials and Methods), and protein complexes were purified by metal affinity chromatography. (*e*) The purification of His_6-Nmd5p was followed for extracts from control and stressed cells, which were supplemented with GST-Gsp1pQ71L (Gsp1p-GTP) as indicated. Comparable amounts of His_6-Nmd5p were isolated for different samples. (*f*) The copurification of Ssa4p(1-236)-GFP with His_6-Nmd5p was quantified for different experimental conditions by measuring the density of bands obtained after ECL (Materials and Methods). The association of Ssa4p(1-236)-GFP with His_6-Nmd5 was determined for three independent experiments, binding of Ssa4p(1-236)-GFP to His_6-Nmd5p in unstressed cells in the absence of GST-Gsp1pQ71L was defined as 100%. The interaction between Ssa4p(1-236)-GFP and His_6-Nmd5p is drastically increased in ethanol stressed cells (x, P=0.04). The formation of His_6-Nmd5p/Ssa4p(1-236)-GFP complexes is significantly reduced by Gsp1p-GTP in control and stressed cells (**, P<0.01). The purification of His_6-Nmd5p was monitored under identical conditions. Similar amounts of the carrier were obtained, independent of stress or the presence of Gsp1p-GTP. Data shown are means and S.D. of three distinct experiments. Samples varied by a maximum of 3 ± 3.7%, relative to the control (no Gsp1p-GTP, no ethanol), which was defined as 100%.

Fig. 10



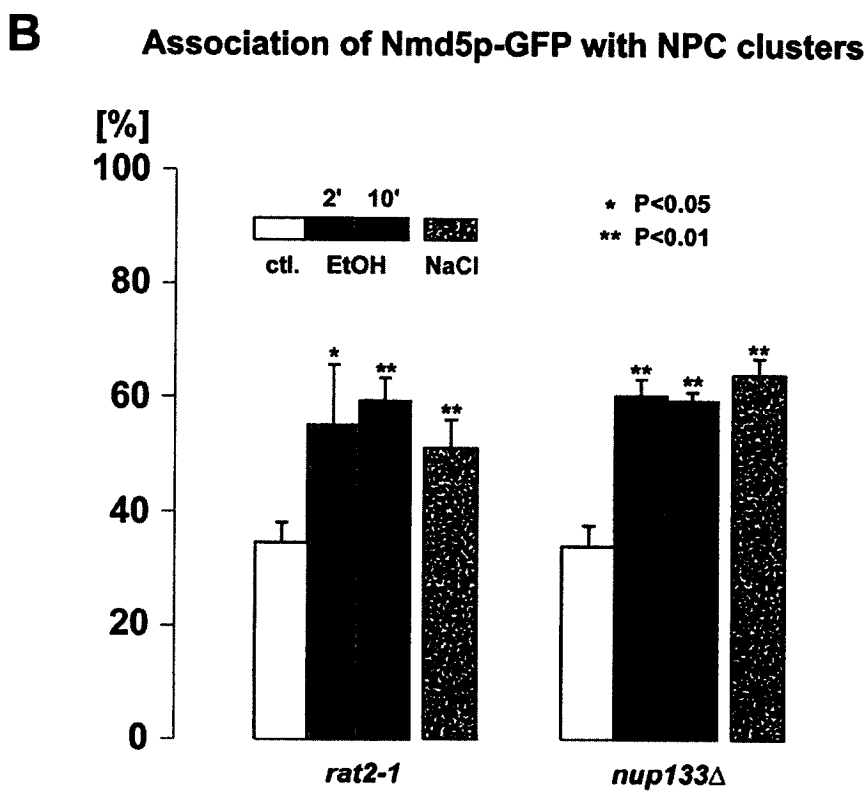


Figure 10. Nmd5p-GFP associates with NPCs in response to ethanol treatment. NPC clustering strains *rat2-1* and *nup133Δ* were analyzed for the distribution of Nmd5p-GFP and NPC clusters under control and stress conditions, that is, 2 min and 10 min 10% ethanol or 10 min 0.4 M NaCl. (*A*) Indirect immunofluorescence with antibodies against GFP and mab414 is shown for *rat2-1* cells. (*B*) Results obtained for *rat2-1* and *nup133Δ* were quantified. At least 100 cells were scored in each of three independent experiments carried out for the different experimental conditions depicted. Means and S.D. are shown.

Fig. 11

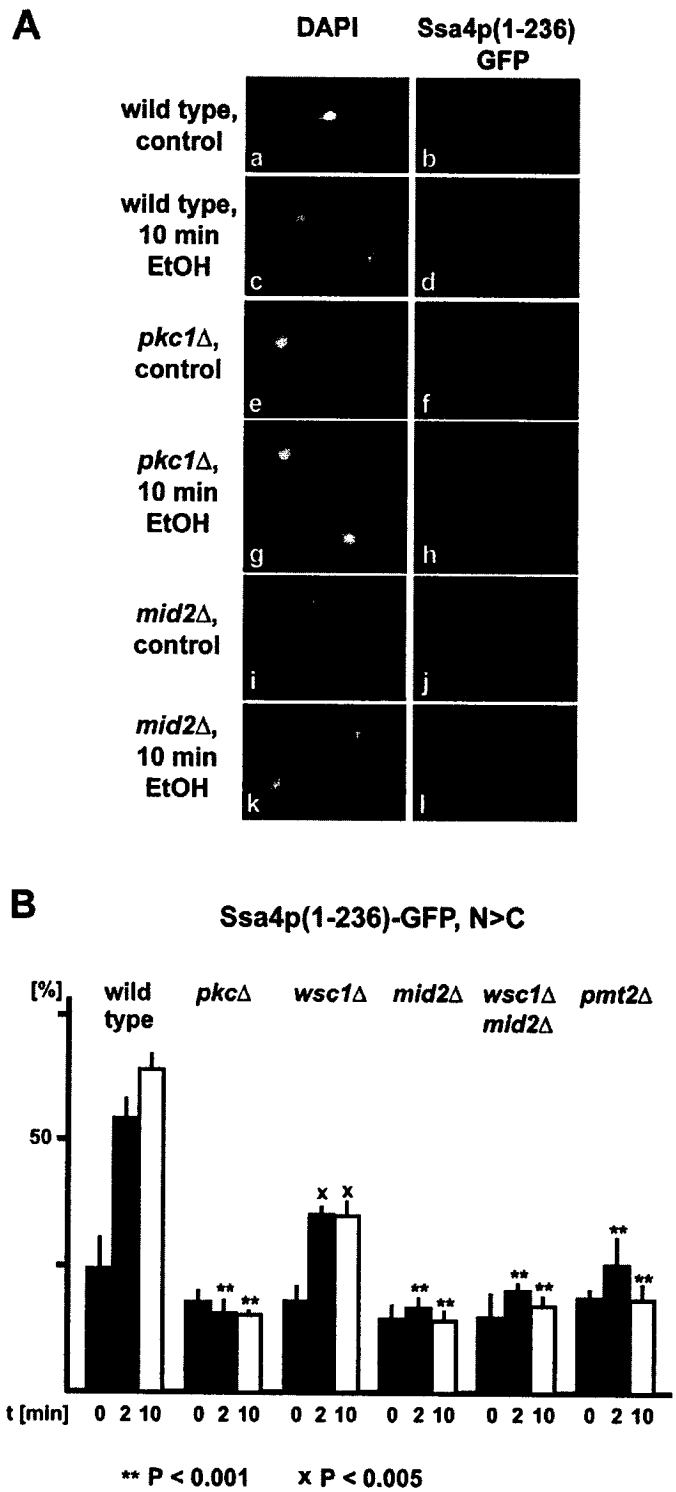


Figure 11. Nuclear accumulation of Ssa4p(1-236)-GFP in ethanol-stressed cells requires components of the cell integrity pathway. (*A*) The accumulation of Ssa4p(1-236)-GFP in nuclei of yeast strains lacking components involved in cell integrity signaling was analyzed under nonstress conditions (control) or after exposure to 10% ethanol for 10 min. (*B*) At least 100 cells were monitored in each of three separate experiments. Nuclear accumulation was scored in unstressed cells (0 min) and upon incubation with 10% ethanol for 2 or 10 min. Results obtained for mutants were compared with those of parental wild-type cells. Means and S.D. are depicted for each strain tested (*x*, $P<0.005$; **, $P<0.001$).

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The carrier Msn5p/Kap142p promotes nuclear export of the hsp70 Ssa4p and relocates in response to stress

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Summary

Cytoplasmic hsp70s like yeast Ssa4p shuttle between nucleus and cytoplasm under normal growth conditions but accumulate in nuclei upon stress. This nuclear accumulation is only transient, and Ssa4p relocates to the cytoplasm when cells recover. We show here that Ssa4p nuclear export is independent of Xpo1/Crm1 and identify the importin-β family member Msn5p/Kap142p as the exporter for Ssa4p. In growing cells and *in vitro*, Msn5p and Ssa4p generate genuine export complexes that require Ran/Gsp1p-GTP. Furthermore, nucleoporin Nup82p, which plays a role in Msn5p-mediated transport, is necessary for efficient export of Ssa4p. In living cells, stress not only regulates Ssa4p localization, but also controls the distribution of Msn5p. Msn5p is concentrated in nuclei of unstressed cells, but appears in the cytoplasm upon exposure to ethanol, heat, starvation or severe oxidative stress. In addition, growth on non-fermentable carbon sources relocates a portion of Msn5p to the cytoplasm and leads to a partial nuclear accumulation of Ssa4p. Taken together, growth and stress conditions that localize the transporter Msn5p to the cytoplasm also induce the nuclear accumulation of its cargo Ssa4p.

Introduction

Cytoplasmic hsp70s shuttle in and out of the nucleus in higher eukaryotes as well as in the budding yeast *Saccharomyces cerevisiae* (Mandell and Feldherr, 1990; Quan *et al.*, 2004; Kodiha *et al.*, 2005). As hsp70s are too large to translocate through the nuclear pore complex (NPC) by diffusion, their transport across the nuclear envelope is likely to be an active process, which may depend on carrier proteins. In yeast, members of the Ssa and Ssb families of hsp70s are present in the cytoplasm, and Ssb1p is excluded from the nucleus due to the presence of a nuclear export signal (NES) that is recognized by Xpo1p (Shulga *et al.*, 1999), the orthologue of metazoan Crm1 (reviewed in Ström and Weis, 2001). In contrast to Ssb1p, Ssa proteins are located in both the cytoplasm and nuclei under non-stress conditions; they accumulate in nuclei when cells are exposed to various insults (Chughtai *et al.*, 2001; Quan *et al.*, 2004). As such, the shuttling protein Ssa4p concentrates in nuclei when cells are exposed to ethanol, and we have identified the importin-β Nmd5p/Kap119p as the carrier that translocates Ssa4p into the nucleus of ethanol-treated cells (Quan *et al.*, 2004). At steady state, the localization of a shuttling protein can be controlled by different mechanisms, which may include changes in both nuclear import and export. Our previous studies have shown that in response to ethanol exposure Ssa4p nuclear import is upregulated on two different levels. First, the formation of import complexes containing Nmd5p and Ssa4p increases; second, docking of Nmd5p at the NPC is enhanced (Quan *et al.*, 2004). The subsequent concentration of Ssa4p in nuclei is only transient, and after removal of ethanol the chaperone relocates to the cytoplasm by an unknown mechanism.

Nuclear transport of most proteins relies on members of the importin-β family. In *S. cerevisiae*, 14 importin-β like carriers have been identified (reviewed in Wozniak *et al.*, 1998; Ström and Weis, 2001; Mosammaparast and Pemberton, 2004) that translocate a large number of cargos through the NPC. Nuclear export mediated by these carriers requires Ran/Gsp1p in its GTP-bound form, which associates with the carrier/cargo complex prior to its translocation across the nuclear envelope. Upon arrival in the cytoplasm Rna1p, the GTPase-activating protein for Gsp1p, stimulates the formation of Gsp1p-GDP, thereby promoting the disassembly of export complexes (reviewed in Weis, 2003; Mosammaparast and Pemberton, 2004).

In budding yeast, four members of the importin-β family participate in nuclear export. The carrier Xpo1p/Crm1 (Stade *et al.*, 1997) moves a large number of proteins out of the nucleus, and NESs recognized by Xpo1p are characterized by the presence of hydrophobic amino acid residues, in particular leucine (reviewed in Kutay and Güttinger, 2005). In higher eukaryotes, Crm1 is inhibited

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by the drug leptomycin B (LMB), a component that covalently modifies the carrier (Kudo *et al.*, 1999). Although Xpo1p in *S. cerevisiae* is not affected by LMB, a LMB-sensitive variant of Xpo1p has been generated that can functionally replace the wild-type protein (Neville and Rosbash, 1999). Cse1p, another importin-β family member, exports selectively Srp1p, a subunit of the classical nuclear import receptor Srp1p/Kap95p (Hood and Silver, 1998; Solsbacher *et al.*, 1998; Takano *et al.*, 2005), whereas Los1p contributes to nuclear export of tRNA (Hellmuth *et al.*, 1998; Sarkar and Hopper, 1998; Steiner-Mosonyi and Mangroo, 2004). Unlike Xpo1p, Cse1p and Los1p, the carrier Msn5p/Kap142p is not only involved in nuclear export, but also participates in protein import. Among the Msn5p export cargos identified so far are several proteins that play a role in signalling, cell cycle control and adaptation to changes in the growth medium (Alepuz *et al.*, 1999). For example, Msn5p exports Far1p, Pho4p, Crz1p, Ste5p, HO endonuclease and Mig1p, a protein involved in the repression of glucose-regulated genes (Kaffman *et al.*, 1998; Blondel *et al.*, 1999; DeVit and Johnston, 1999; Mahanty *et al.*, 1999; Boustany and Cyert, 2002; Kaplun *et al.*, 2003; Queralt and Igual, 2003). Export of several Msn5p cargos is regulated by the growth conditions; in particular, the availability of nutrients like glucose and phosphate plays a role (reviewed in Hood and Silver, 1999). In addition to export, Msn5p was reported to promote nuclear import of replication protein A (Yoshida and Blobel, 2001). In higher eukaryotes Msn5p-like carriers may have more complex functions, as the *Arabidopsis* Msn5p orthologue *HASTY* participates in the control of plant development (Bollman *et al.*, 2003).

Although the effect of different physiological conditions on the localization of several Msn5p cargos has been characterized previously, it has yet to be determined whether changes in cell physiology also affect the distribution of the carrier. We show now that in *S. cerevisiae* Msn5p promotes nuclear export of the chaperone Ssa4p and that the localization of Msn5p is sensitive to stress and regulated by the carbon source. We propose that the distribution of Msn5p under different growth and stress conditions may contribute for some cargos to the regulation of Msn5p-mediated export.

Results

We have previously shown that the Ssa4p N-terminal portion and Ssa4p behave similar with respect to nuclear import (Quan *et al.*, 2004). Furthermore, Ssa4p contains a peptide binding domain in its C-terminal part, which is required to bind substrates that need folding/refolding. Interactions of the peptide binding domain with folding substrates can lead to retention within a cellular compartment, thereby preventing nucleocytoplasmic trafficking (Kodiha *et al.*, 2005). To analyse the role of carriers in Ssa4p nuclear transport, we were particularly interested in studying the N-terminal portion of Ssa4p.

The nuclear exporter Xpo1p/Crm1 is not essential for transport of Ssa4p to the cytoplasm

The carrier Xpo1p is involved in export of a large number of cargos, and NESs that bind Xpo1p are rich in hydrophobic amino acid residues, such as leucine (reviewed in Kutay and Güttinger, 2005). A sequence matching the consensus of an Xpo1p-specific NES is present in the N-terminal domain of Ssa4p and we have tested the role of Xpo1p in export of Ssa4p(1-236)-GFP. To this end, two different strains that are mutated in *XPO1* were analysed (Fig. S1A and B). We first monitored the distribution of Ssa4p(1-236)-GFP in a mutant yeast strain that carries a LMB-sensitive allele *XPO1 T539C* (Neville and Rosbash, 1999). The fluorescent reporter protein NES-NLS-GFP2, which was used in control experiments, shuttles between nucleus and cytoplasm and is exported to the cytoplasm by Xpo1p (Stade *et al.*, 1997). In *XPO1 T539C* cells NES-NLS-GFP2 rapidly accumulates in nuclei upon addition of LMB (Neville and Rosbash, 1999 and Fig. S1A). By contrast, LMB failed to concentrate Ssa4p(1-236)-GFP in nuclei and no significant difference was detected when the localization of Ssa4p(1-236)-GFP in untreated controls, cells treated with solvent only or LMB was quantified (Fig. S1A).

In addition, a strain carrying the temperature-sensitive allele *xpo1-1* was exposed to 37°C for 15 or 30 min to inactivate xpo1-1p, and Ssa4p(1-236)-GFP was localized before and after heat treatment. Heat inactivation of xpo1-1p did not result in nuclear accumulation of the reporter protein, which was nuclear and cytoplasmic in *xpo1-1* and isogenic *XPO1* cells (Fig. S1B). After a 30 min exposure to 37°C, Ssa4p(1-236)-GFP was nuclear as well as cytoplasmic and in part present in large aggregates in both mutant and wild-type cells. *xpo1-1* and *XPO1* cells showed similar localization of Ssa4p(1-236)-GFP after incubation at room temperature or 37°C. Taken together, these results are in line with the idea that Xpo1p does not play a major role in Ssa4p(1-236)-GFP nuclear export.

Ssa4p concentrates in nuclei of unstressed cells that contain a deletion of MSN5

We have previously localized Ssa4p(1-236)-GFP in different yeast strains that carry a deletion of one of the importin-β genes. These studies identified the carrier Nmd5p as an importer for Ssa4p(1-236)-GFP in ethanol-treated cells. Under non-stress conditions, all of the deletion mutants analysed so far located Ssa4p(1-236)-GFP to the nucleus and cytoplasm (Quan *et al.*, 2004 and Fig. 1). Unlike other importin-β mutants, exemplified by

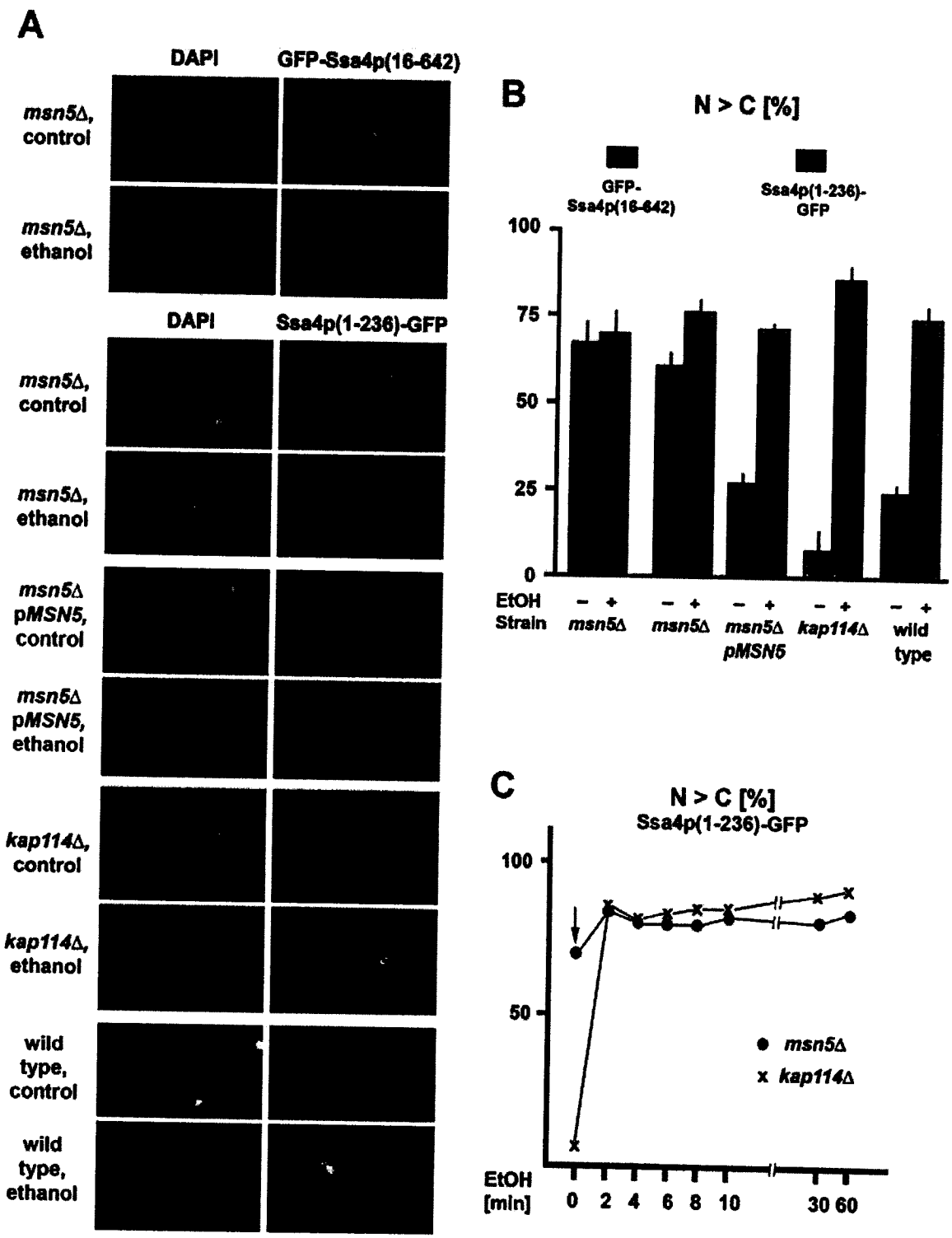

Fig. 1. Nuclear export of Ssa4p is mediated by the importin-β Msn5p.
A. Yeast strain *msn5Δ* was analysed for the distribution of GFP-Ssa4p(16-642) or Ssa4p(1-236)-GFP under non-stress conditions and upon addition of ethanol. Wild-type *MSN5* encoded by an episomal plasmid (p*MSN5*) was introduced into strain *msn5Δ* followed by localization of Ssa4p(1-236)-GFP. For comparison, the importin-β mutant *kap114Δ* and wild-type strain W303 were analysed under identical conditions.
B. The localization of fluorescent reporter proteins was determined in at least 100 cells. Means and standard deviations are shown for at least three independent experiments.
C. Nuclear accumulation of Ssa4p(1-236)-GFP was monitored at different time points in *msn5Δ* and *kap114Δ* cells. Note that under non-stress conditions at 0 min elevated levels of Ssa4p(1-236)-GFP are present in nuclei (indicated by an arrow).

*kap114*Δ in Fig. 1, *msn5*Δ had elevated levels of Ssa4p(1-236)-GFP in the nucleus even under normal growth conditions (Fig. 1A–C, arrow). Similar to the N-terminal 236 residues of Ssa4p, the fusion protein GFP-Ssa4p(16-642) accumulated also in the nucleus of unstressed *msn5*Δ cells (Fig. 1A and B). This mislocalization of Ssa4p(1-236)-GFP could be rescued by introduction of a wild-type copy of *MSN5* into *msn5*Δ cells. In cells that contained *MSN5* on an episomal plasmid (p*MSN5*), Ssa4p(1-236)-GFP was nuclear and cytoplasmic under non-stress conditions. Upon treatment with ethanol Ssa4p(1-236)-GFP accumulation in nuclei resembled wild-type cells (Fig. 1A and B). Similar results were obtained when experiments were repeated with *MSN5* introduced into cells on a centromeric plasmid (Fig. S2).

When *msn5*Δ cells were incubated with 10% ethanol, the number of cells that concentrated Ssa4p(1-236)-GFP in nuclei was further increased when compared with unstressed controls. The kinetics of ethanol-induced nuclear accumulation was similar in *msn5*Δ, wild-type cells or strains lacking other importin-β genes, and the maximum concentration in nuclei was reached after a 2 min ethanol treatment (Fig. 1C). This increase of Ssa4p(1-236)-GFP nuclear accumulation in *msn5*Δ cells upon addition of ethanol is likely due to the upregulation of nuclear import (Quan *et al.*, 2004), a process that is independent of the exporter *MSN5*.

The results for importin-β mutants described above are consistent with the idea that Msn5p participates in Ssa4p nuclear export and prompted us to further define the possible role of this carrier in Ssa4p nuclear trafficking.

Nuclear transport of Ssa4p(1-236)-GFP requires the nucleoporin Nup82p

The essential nucleoporin Nup82p is located at the cytoplasmic side of the NPC, where it participates in Msn5p-mediated export and other nuclear transport reactions (Belanger *et al.*, 2004). Moreover, Nup82p can directly interact with Msn5p (Damelin and Silver, 2000). We therefore tested whether Nup82p is involved in Ssa4p(1-236)-GFP transport as well. Control and mutant cells were incubated for 1 h at 37°C, which will inactivate the temperature-sensitive *nup82-3* protein. When compared with wild-type cells *nup82-3* was less efficient in concentrating Ssa4p(1-236)-GFP in nuclei in response to ethanol exposure. This was seen both at room temperature and 37°C, but the defect was more severe in *nup82-3* cells that had been pre-incubated at the non-permissive temperature (Fig. 2A). The addition of ethanol resulted in the reversible formation of aggregates for some cells, which disappeared after a 1 h recovery period (not shown).

To determine a possible contribution of *nup82-3* to Ssa4p(1-236)-GFP export, all steps were carried out at the non-permissive temperature. Wild-type (W303) and mutant strains pre-incubated for 1 h at 37°C were treated for 10 min with 10% ethanol, and the percentage of cells that had accumulated the reporter protein in nuclei was given the arbitrary value of 1. Following ethanol exposure, cells were transferred to ethanol-free medium to monitor the relocation of Ssa4p(1-236)-GFP to the cytoplasm. When compared with wild-type cells treated under identical conditions, *nup82-3* was less efficient in relocating Ssa4p(1-236)-GFP to the cytoplasm (Fig. 2B). In addition to *nup82-3*, Ssa4p(1-236)-GFP nuclear export was tested in other strains that carry a mutation in a different nucleoporin gene. To this end, temperature-sensitive mutants *nup1-8* and *nsp1-5* were treated as described for *nup82-3*. Both *nup1-8* and *nsp1-5* were similar to wild-type cells with respect to nuclear export of Ssa4p(1-236)-GFP after recovery from ethanol stress (Fig. 2B). In summary, these results suggest that Nup82p participates both in nuclear import and export of Ssa4p. The role of Nup82p in Ssa4p transport is specific as export inhibition was not detected for other nucleoporin mutations that were analysed under the same conditions.

Ssa4p(1-236)-GFP generates export complexes with Msn5p in growing cells and in vitro

To characterize the role of Msn5p in nuclear transport of Ssa4p we tested whether both proteins can generate export complexes in living cells. Myc-tagged Msn5p integrated into the chromosome (Alepuz *et al.*, 1999) was immunoprecipitated from crude cell extracts and purified material was probed with antibodies against GFP. Under these conditions, GFP-Ssa4p(16-642) and Ssa4p(1-236)-GFP, but not GFP, co-purified with Myc-Msn5p (Fig. 3A). As Ssa4p(1-236)-GFP is missing the peptide-binding site of the hsp70, its association with Msn5p is unlikely to represent a simple chaperone/folding substrate interaction. Furthermore, the formation of nuclear export complexes containing members of the importin-β family requires Gsp1p-GTP, whereas chaperone/unfolded protein interactions do not depend on Gsp1p. The importance of Gsp1p-GTP was evaluated by adding the GTPase-activating protein Rna1p to crude extracts before purifying Msn5p/Ssa4p complexes. Stimulation of the Gsp1p-GTPase activity by Rna1p will lower the concentration of Gsp1p-GTP, thereby interfering with export complex formation. As shown in Fig. 3A, the addition of Rna1p drastically reduced the amount of GFP-Ssa4p(16-642) or Ssa4p(1-236)-GFP that co-purified with the carrier, supporting the idea that Gsp1p-GTP stimulates the association of Msn5p and Ssa4p.

In order to determine whether Ssa4p and Msn5p can interact directly, binding was tested with purified compo-

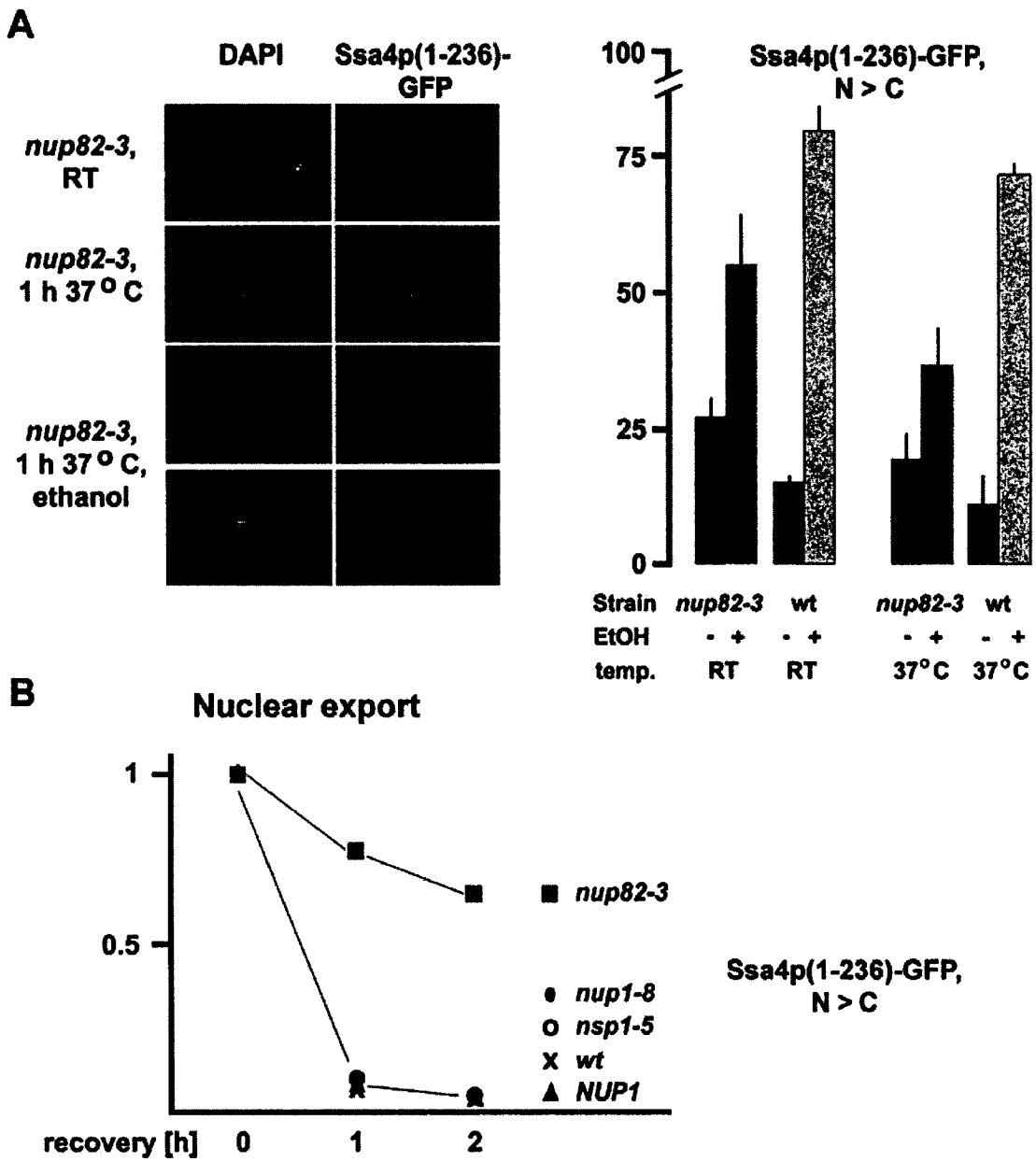

Fig. 2. The nucleoporin Nup82p is involved in nuclear transport of Ssa4p(1-236)-GFP.
A. Wild-type and *nup82-3* cells were kept at room temperature (RT) or incubated for 1 h at 37°C and subsequently treated for 10 min with 10% ethanol. Ssa4p(1-236)-GFP was localized by fluorescence microscopy. The distribution of Ssa4p(1-236)-GFP was quantified as in Fig. 1.
B. Export of Ssa4p(1-236)-GFP to the cytoplasm is compromised in *nup82-3* mutants at the non-permissive temperature. The number of wild-type or mutant cells, respectively, that show nuclear accumulation of Ssa4p(1-236)-GFP after ethanol treatment was defined as 1. After removal of ethanol, aliquots of the cultures were fixed and the distribution of Ssa4p(1-236)-GFP was quantified. For comparison, nucleoporin mutants *nup1-8* and *nsp1-5* and wild-type strains (W303, wt; *NUP1*) were analysed under the same conditions. Data are shown for three independent experiments; at least 100 cells were scored for each data point and experiment.

nents *in vitro* using immobilized metal affinity chromatography and His6-tagged Ssa4p proteins as a bait. In the absence of Gsp1p-GTP we detected only little association of Ssa4p(1-236)-GFP with the carrier Msn5p (Fig. 3B). However, this interaction was increased to 5.5-fold by the addition of Gsp1p-GTP (Fig. 3B). In control experiments, non-specific binding of Msn5p or GST to the resin was negligible and Gsp1p-GDP had no effect on the formation of transport complexes. We further tested His6-tagged Ssa4p in pull-down experiments under identical conditions (Fig. 3C). Like the N-terminal portion of Ssa4p, His6-Ssa4p(16-642) binds Msn5p *in vitro*, and the interaction is

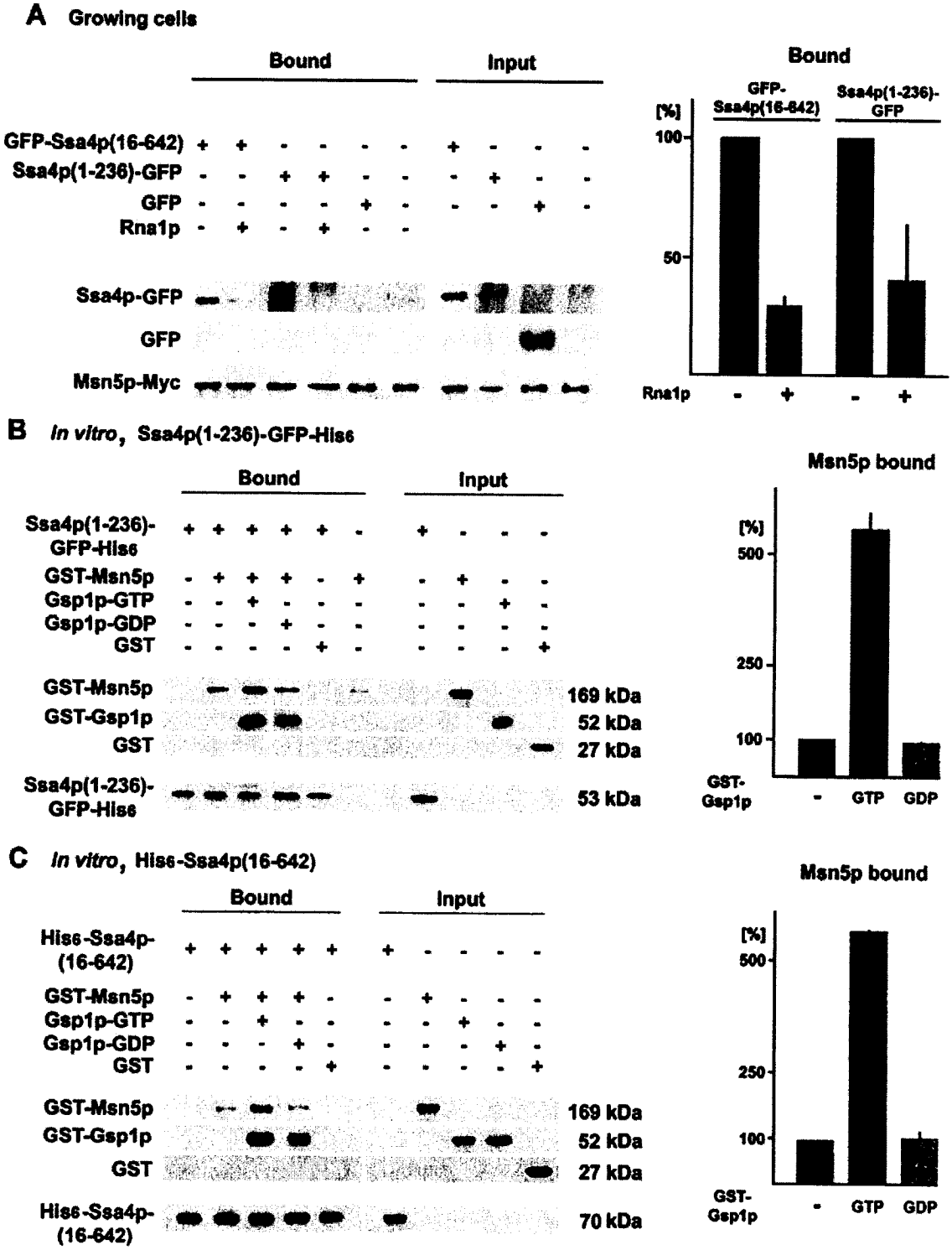

Fig. 3. Ssa4p(1-236)-GFP associates with Myc-tagged Msn5p in growing cells and *in vitro*.
A. Msn5p-containing protein complexes were purified from growing cells as described in *Experimental procedures* and analysed by Western blotting with antibodies against GFP or the Myc-epitope.
B. Pull-down experiments were carried out with purified Ssa4p(1-236)-GFP-His6, GST-Msn5p, GST-Gsp1pQ71L (Gsp1p-GTP) or GST-Gsp1p (Gsp1p-GDP) as shown in the figure. GFP-His6 was used in control experiments.
C. The experiments shown in part B were carried out with His6-Ssa4p(16-642). The amounts of GFP-Ssa4p(16-642) and Ssa4p(1-236)-GFP bound to Msn5p-Myc (part A) or Myc-tagged Msn5p co-purifying with Ssa4p (parts B, C) were measured after Western blotting by densitometry of the film (*Experimental procedures*).

stimulated to 5.7-fold binding in the presence of Gsp1p-GTP. Thus, both proteins generate genuine nuclear export complexes with Msn5p in growing cells and *in vitro*.

Msn5p localization is regulated by the exposure to stress, but independent of the presence of cargo

Several stressors promote the nuclear accumulation of Ssa4p, whereas exposure to osmolytes or mild oxidative stress is not efficient in concentrating the chaperone in nuclei (Chughtai *et al.*, 2001; Quan *et al.*, 2004). Like ethanol treatment or starvation (Chughtai *et al.*, 2001; Quan *et al.*, 2004), we have identified heat shock (6 h at 37°C or 1 h at 42°C) as another form of stress that accumulates Ssa4p(1-236)-GFP in nuclei (Fig. 4C). To determine whether environmental changes that redistribute Ssa4p(1-236)-GFP also affect Msn5p, the carrier was localized in control and stressed cells. Msn5p accumulated in nuclei under control conditions, but appeared in the cytoplasm in response to ethanol, heat, starvation or severe oxidative stress (Fig. 4A, N + C). When different types of stress were compared, starvation was the most effective and all of the starving cells redistributed Msn5p. By contrast, exposure to osmotic or mild oxidative stress had no severe effects. Likewise, these treatments do not alter drastically the localization of Ssa4p (Quan *et al.*, 2004). The stress-induced changes in the distribution of Msn5p do not simply reflect degradation of the carrier. Western blot analysis of crude extracts prepared from control and treated cells revealed that the levels of Msn5p were similar under all conditions tested (Fig. 4B and data not shown).

One possible explanation for the concentration of Msn5p in unstressed cells could be a retention process whereby the carrier binds to cargo in the nucleus. For instance, cargo associated with chromatin or other nuclear structures might prevent the carrier from moving to the cytoplasm. A mislocalization of Msn5p could then be explained by the absence of cargo from the nucleus. If this was the case, one would predict that a reduction of cargo in nuclei will lead to an increase of cytoplasmic Msn5p levels. To begin to address this question, we compared the localization of Msn5p-GFP in wild-type cells and mutants *pho4*Δ or *far1*Δ (Fig. 4D). Msn5p-GFP was concentrated in nuclei for all strains tested, and no drastic changes were detected in mutant cells. This suggests that the deletion of either cargo does not alter Msn5p distribution; however, we cannot rule out that other cargos might contribute to the nuclear retention of Msn5p.

Stress controls the formation of Msn5p export complexes

In addition to the mislocalization of Msn5p to the cytoplasm, other mechanisms may prevent Ssa4p nuclear export upon stress. One level of regulation could be the formation of export complexes containing Msn5p and Ssa4p(1-236)-GFP. To test this hypothesis, pull-down experiments were carried out with crude extracts from cells that had been exposed to different stresses (Fig. 5). Exposure of cells to heat, starvation or ethanol drastically reduced the association between carrier and cargo, supporting the idea that these conditions reduce the formation of export complexes.

Ethanol-induced relocation of Msn5p is reversible during stress recovery

Nuclear accumulation of the Msn5p-cargo Ssa4p(1-236)-GFP in ethanol-treated cells is reversible, and Ssa4p(1-236)-GFP migrates back to the cytoplasm when ethanol is removed. This export to the cytoplasm is independent of *de novo* protein synthesis (Quan *et al.*, 2004). To analyse whether the Ssa4p exporter Msn5p also redistributes during recovery, cells were treated for 10 min with 10% ethanol and subsequently transferred to ethanol-free medium. Removal of ethanol induced the re-accumulation of Msn5p in nuclei. This process does not require protein synthesis, as it was insensitive to cycloheximide (Fig. 6A and B). The kinetics of Msn5p and Ssa4p(1-236)-GFP relocation during stress recovery suggests that increasing the concentration of Msn5p in nuclei correlates with the appearance of Ssa4p(1-236)-GFP in the cytoplasm (Fig. 6A and B).

Msn5p and Ssa4p(1-236)-GFP localization is regulated by the carbon source

Msn5p is involved in nuclear export of proteins that regulate glucose-dependent gene expression, such as Mig1p, and we tested whether growth on fermentable versus non-fermentable carbon sources affects the localization of Msn5p. As reported previously (Alepuz *et al.*, 1999), the carrier is concentrated in nuclei of cells growing in rich medium supplemented with glucose. However, when cells were grown on YP-medium containing glycerol or ethanol approximately half of the cells showed Msn5p in the cytoplasm (data not shown). Similar results were obtained when minimal medium was used instead of YP (Fig. 7A). Moreover, like Msn5p the distribution of Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) were also regulated by the carbon source, and increased amounts of the reporter protein were detected in nuclei whenever elevated levels of Msn5p were in the cytoplasm (Fig. 7A).

In control experiments, the Mig1p transport domain (residues 261–400; DeVit *et al.*, 1997) fused to GFP-β-galactosidase, here referred to as Mig1p-GFP-β-gal, was analysed (Fig. 7A). Mig1p controls the expression of

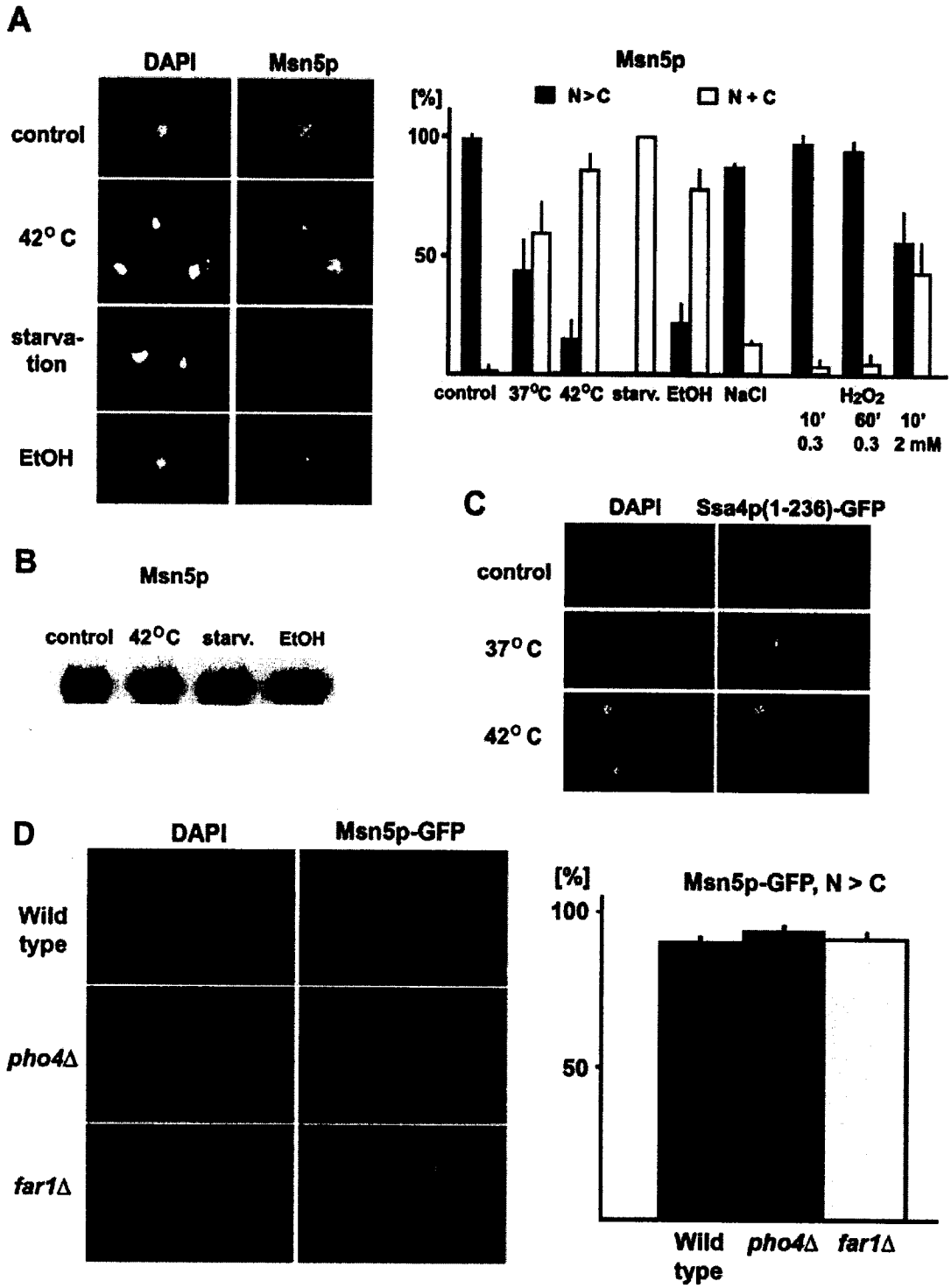

Fig. 4. Localization of Msn5p is sensitive to several forms of stress. Myc-tagged Msn5p was localized in yeast cells exposed to heat (6 h at 37°C, 1 h at 42°C), starvation, ethanol (10 min, 10%), NaCl (10 min, 0.4 M) or H_2O_2 (10 min, 0.3 mM; 60 min, 0.3 mM; 10 min, 2 mM).
A. Myc-Msn5p was detected by indirect immunofluorescence and results were quantified as in Fig. 1.
B. Protein extracts were prepared for controls or cells exposed to heat (1 h at 42°C), starvation or ethanol (10 min, 10%). Comparable amounts of protein were probed with antibodies against the Myc-epitope.
C. Ssa4p(1-236)-GFP accumulates in nuclei of heat-stressed cells. Ssa4p(1-236)-GFP was localized in controls and cells incubated for 6 h at 37°C or 1 h at 42°C.
D. Msn5p-GFP was localized in wild-type cells or strains *pho4Δ* and *far1Δ*. Results are shown for three separate experiments, for each at least 100 cells were scored.

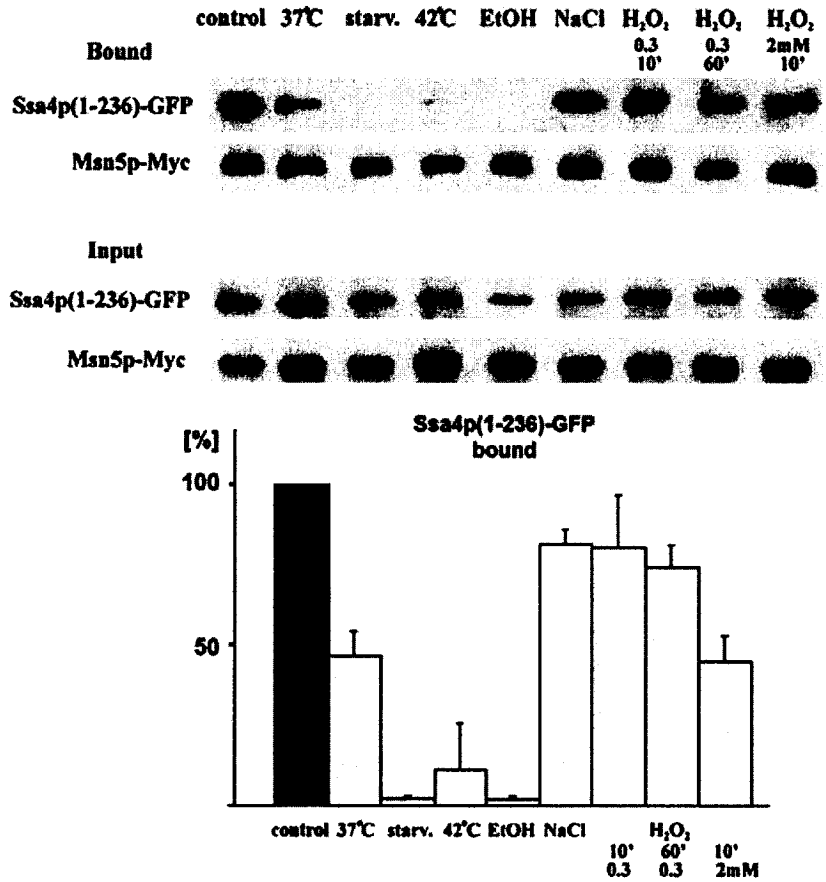

Fig. 5. Stress regulates the interaction between Msn5p and Ssa4p(1-236)-GFP. Msn5p-containing complexes were isolated from growing cells and binding was quantified as in Fig. 3A. The amount of Ssa4p(1-236)-GFP bound under control conditions was defined as 1. Means and standard deviations are shown for at least three independent experiments for each condition.

glucose-regulated genes, and nucleocytoplasmic trafficking of Mig1p mediated by the transport domain is crucial to this process (DeVit *et al.*, 1997; DeVit and Johnston, 1999). Msn5p is the nuclear exporter for Mig1p, and the nuclear accumulation of Mig1p depends on the availability of glucose (DeVit *et al.*, 1997). As expected, in glucose-containing medium, Mig1p-GFP-β-gal is restricted to nuclei, but mostly cytoplasmic when cells were provided with 3% glycerol or 2% ethanol as a carbon source.

To test in more detail how a shift from fermentable to non-fermentable carbon source affects Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) localization, cells were grown overnight in minimal medium containing 2% galactose and transferred to medium containing either glycerol or ethanol (Fig. 7B). After 1 h incubation in medium containing glycerol or ethanol, nuclear concentration of Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) was observed in 77–79% and 71–73% of the cells respectively. After continued growth on glycerol or ethanol nuclear accumulation was maintained at a level of 54–58% for Ssa4p(1-236)-GFP and 48–49% for GFP-Ssa4p(16-642).

Different mechanisms control nuclear export of the transcription factor Mig1p and the hsp70 Ssa4p

Additional control studies were carried out to determine whether stress alters in general the distribution of Msn5p-cargos. To this end, the localization of Mig1p-GFP-β-gal, Pho4p-GFP and Far1p-GFP was monitored in cells exposed to heat, ethanol for 10 min, starvation, osmotic or oxidative stress (Figs 8 and 9). Starvation was very efficient in relocating Mig1p-GFP-β-gal to the cytoplasm, whereas none of the other treatments prevented nuclear accumulation of Mig1p-GFP-β-gal. Although some of the cells exposed to 10% ethanol showed faint cytoplasmic staining, most of Mig1p-GFP-β-gal remained concentrated in nuclei in response to 10 min ethanol stress (Fig. 8). Unlike Mig1p, Pho4p-GFP and Far1p-GFP were efficiently relocated to the cytoplasm upon exposure to heat, starvation and ethanol (Fig. 9).

In summary, the distribution of Ssa4p(1-236)-GFP correlated with the location of Msn5p; whenever Msn5p was detected in the cytoplasm, elevated levels of Ssa4p(1-236)-GFP appeared in the nucleus. No such link could be detected for Msn5p and Mig1p-GFP-β-gal, whereas

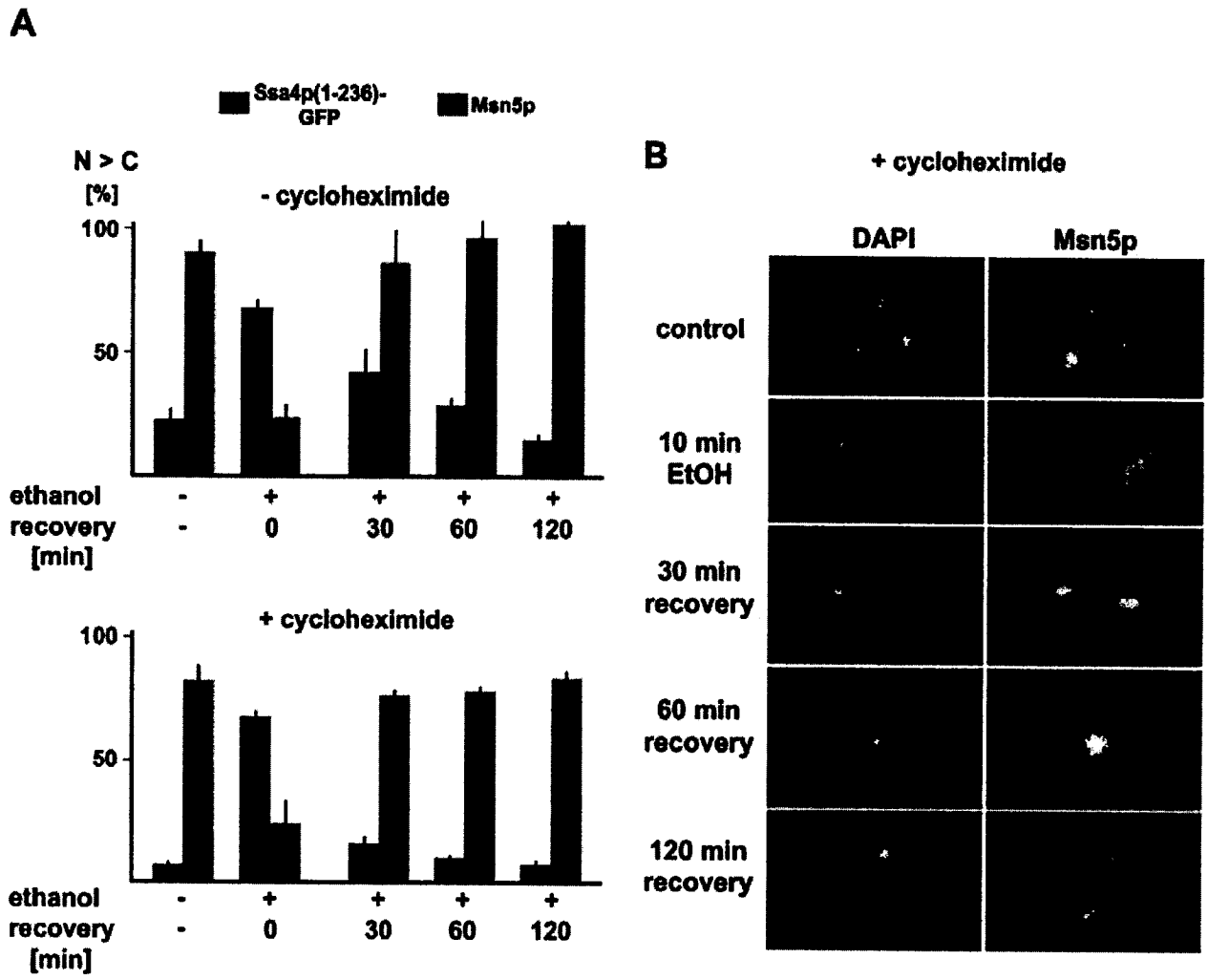

Fig. 6. Msn5p localizes to the cytoplasm upon ethanol treatment and relocates to the nucleus during recovery.
A. Yeast cells synthesizing Ssa4p(1-236)-GFP and Myc-tagged Msn5p were kept under non-stress conditions (– ethanol, – recovery) or treated for 10 min with 10% ethanol. Cells recovered in ethanol-free medium for the times indicated. Ssa4p(1-236)-GFP and Msn5p-Myc were located for the different time points by fluorescence microscopy or indirect immunofluorescence. The localization of Ssa4p(1-236)-GFP and Msn5p-Myc was quantified as described for Fig. 1. In the lower panel cycloheximide was present at 100 μg ml^{-1} throughout the stress and recovery period.
B. The localization of Msn5p-Myc was monitored in control, ethanol-stressed and recovering cells by indirect immunofluorescence with antibodies against the Myc-epitope.

Pho1p-GFP and Far1p-GFP were also mislocalized in response to heat, starvation and ethanol. In all cases, starvation had the strongest effect on relocating Msn5p and its cargos.

Mutant msn5Δ *and* nmd5Δ *cells are more sensitive to stress than wild-type cells*

Although *MSN5* and *NMD5* are not essential genes it was possible that either carrier improves the survival of stressed cells. We therefore tested the viability of strains lacking *MSN5* or *NMD5* after exposure to different stresses and compared them with the wild-type strain W303, which has the same genetic background as both mutants (Fig. 10). Survival rates were measured as the number of colonies formed on plates, and survival of W303 was defined as 100% for each of the stress conditions tested. For heat, starvation, ethanol and 1 h exposure to oxidative stress *msn5Δ* and *nmd5Δ* had lower survival rates than wild-type cells, suggesting an important role of either carrier in the proper response to some forms of stress.

Ssa4p protects yeast cells from stress

To evaluate the possible contribution of Ssa4p to stress survival, yeast cells that carry the *SSA1* gene as the only

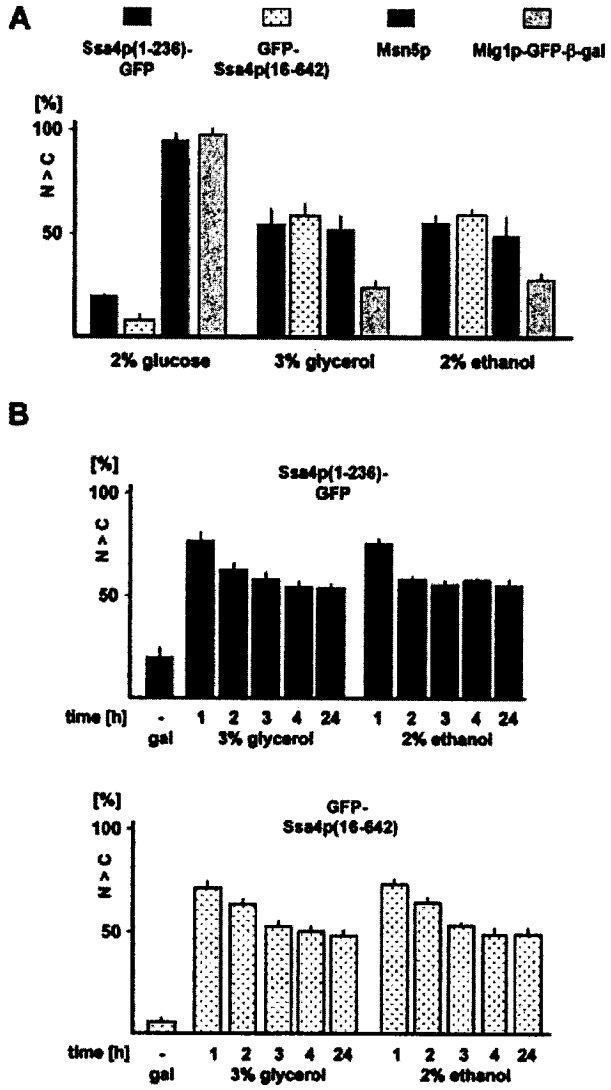

Fig. 7. Non-fermentable carbon sources alter the distribution of Ssa4p(1-236)-GFP, GFP-Ssa4p(16-642) and Msn5p.
A. Yeast cells synthesizing Ssa4p(1-236)-GFP, GFP-Ssa4p(16-642), Myc-tagged Msn5p or Mig1p(261–400)-GFP-β-galactosidase [Mig1p-GFP-β-gal] were grown overnight in minimal medium supplemented with 2% glucose, 3% glycerol or 2% ethanol. Msn5p and GFP-containing reporter proteins were localized in three independent experiments; at least 100 cells were scored in each experiment for the distribution of carrier and cargo.
B. Yeast cells were grown overnight on minimal medium with 2% galactose to induce the synthesis of Ssa4p(1-236)-GFP or GFP-Ssa4p(16-642), collected by centrifugation, washed and resuspended in minimal medium containing 3% glycerol or 2% ethanol. Samples were fixed 1, 2, 3, 4 or 24 h after change of the carbon source and the distribution of Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) was measured as described for part A.

member of the *SSA* family (JN516; Becker *et al.*, 1996) were exposed to heat, starvation, ethanol, osmolytes and oxidants. The survival rates were compared with cells that, in addition to Ssa1p, synthesize GFP-Ssa4p(16-642) or Ssa4p(1-236)-GFP. Cells with the same genetic background but containing all members of the *SSA* family (*SSA1 SSA2 SSA3 SSA4*; JN55; Becker *et al.*, 1996) were used as a wild-type control and the survival of this strain was defined as 100% for each stress analysed. In the absence of *SSA2 SSA3 SSA4* cells were severely affected by stress (Fig. 11); however, overexpression of GFP-Ssa4p(16-642) could rescue this strain and survival was similar to the wild-type control. Some protection against stress was also observed for Ssa4p(1-236)-GFP, but it was always less than for GFP-Ssa4p(16-642). These results are consistent with the idea that the synthesis of Ssa4p helps to protect cells from stress-induced death.

Discussion

Our studies were undertaken to identify and characterize the nuclear exporter for Ssa4p, a chaperone that shuttles between nucleus and cytoplasm. Ssa4p concentrates in nuclei in response to certain environmental changes, and we have previously shown that the importin-β Nmd5p is required for Ssa4p nuclear accumulation in cells that have been exposed to ethanol or starvation (Chughtai *et al.*, 2001; Quan *et al.*, 2004). Specifically, ethanol treatment upregulates nuclear import of Ssa4p(1-236)-GFP by stimulating the formation of Ssa4p(1-236)-GFP/Nmd5p import complexes and docking of Nmd5p at the NPC (Quan *et al.*, 2004).

The results presented here show that Msn5p, a carrier that participates both in nuclear import and export of proteins, translocates Ssa4p from nuclei to the cytoplasm. The steady-state distribution of a shuttling protein is the result of nuclear import, export and retention in either cellular compartment. So far, it is not known whether retention contributes to the intracellular localization of Ssa4p. We have therefore focused on the mechanisms that are involved in Ssa4p transport across the nuclear envelope. Our mutant analyses show that even under non-stress conditions Ssa4p is accumulated in nuclei of *msn5Δ* cells, suggesting that impaired export of the hsp70 increases its nuclear concentration. Moreover, when a wild-type copy of *MSN5* is introduced into the *msn5Δ* mutant Ssa4p redistributes throughout nucleus and cytoplasm in unstressed cells. Thus, we conclude that nuclear export promoted by Msn5p plays a role in the regulation of Ssa4p distribution. Further support for this hypothesis comes from the analysis of *nup82-3*, a mutant nucleoporin that affects Msn5p-mediated export (Belanger *et al.*, 2004). Surprisingly, *nup82-3* cells have an additional defect in ethanol-induced Ssa4p nuclear import. Nup82p does not only participate in Msn5p-dependent trafficking, this nucleoporin is also involved in other nuclear transport pathways, such as classical

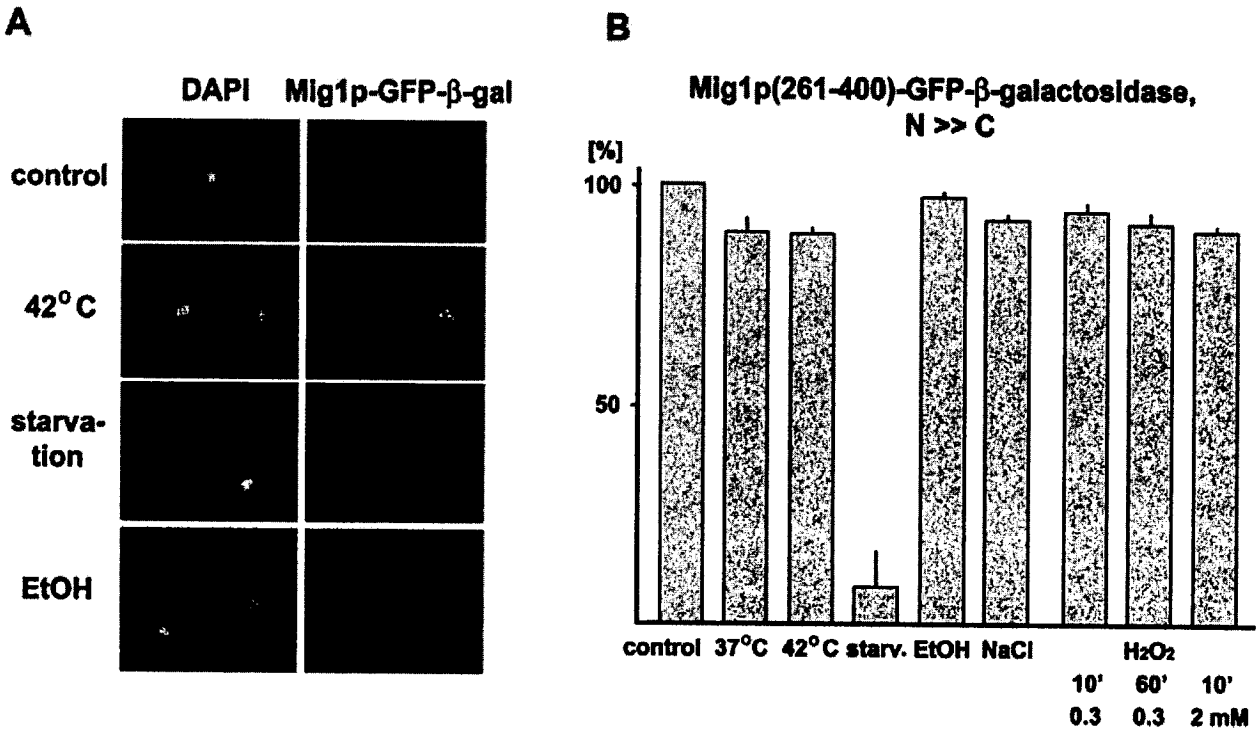

Fig. 8. Mig1p relocates to the cytoplasm of starving cells, but not in response to other stresses. Yeast cells synthesizing Mig1p(261–400)-GFP-β-galactosidase (Mig1p-GFP-β-gal) were incubated for 6 h at 37°C, 1 h at 42°C, starved, treated with 10% ethanol (10 min), 0.3 mM H_2O_2 (10 min, 60 min) or 2 mM H_2O_2 (10 min). Nuclear accumulation of the reporter protein Mig1p-GFP-β-gal was monitored for at least 100 cells in each of three independent experiments. Means and standard deviations are shown. (A) Mig1p(261–400)-GFP-β-galactosidase was located by fluorescence microscopy and results were quantified after exposure to different stressors (B).

nuclear import and interacts with the importin-β-like carrier Pse1p (Damelin and Silver, 2000; Belanger *et al.*, 2004). Based on these data, a possible explanation for the impaired Ssa4p import in *nup82-3* cells could be the direct or indirect interaction of Nup82p with Nmd5p, the carrier that translocates Ssa4p into nuclei of ethanol-treated cells (Quan *et al.*, 2004). A direct role of Msn5p in Ssa4p nuclear export was demonstrated here by the purification of transport complexes from growing cells that contain both the carrier and hsp70. Importantly, the interaction was reduced by the Gsp1p-GTPase-activating protein Rna1p, suggesting that Msn5p and Ssa4p are components of a genuine nuclear export complex that is comprised of carrier/cargo/Gsp1p-GTP. This model is consistent with *in vitro* binding assays, which show that a direct interaction between Msn5p and Ssa4p is stimulated significantly by Gsp1p-GTP but not Gsp1p-GDP. Taken together, the results for growing cells as well as *in vitro* data demonstrate an essential role for Msn5p in Ssa4p nuclear export. Unlike Ssa4p, other cytoplasmic hsp70s may use distinct nuclear carriers for export to the cytoplasm. This is exemplified by Ssb1p, which is excluded from nuclei under steady-state conditions due to its export by Xpo1p (Shulga *et al.*, 1999).

MSN5 or *NMD5* are not essential genes, yeast cells lacking a functional copy of either gene display a lower viability than wild-type strain W303, which has the same genetic background as the carrier mutants. Although Ssa4p is one of the cargos whose nuclear transport is altered by *msn5Δ* and *nmd5Δ*, it is obvious that the absence of Msn5p or Nmd5p will impact a larger number of proteins that move in and out of the nucleus. For instance, the distribution of Msn2p and Hog1p, proteins involved in the response to oxidative and osmotic stress, is regulated by Msn5p and Nmd5p respectively (Ferrigno *et al.*, 1998; Görner *et al.*, 2002). The lack of proper trafficking of cargos other than Ssa4p, which are required for the appropriate response and adaptation to stress, is likely to contribute to the reduced survival of *msn5Δ* and *nmd5Δ* cells. Like Msn5p and Nmd5p, Ssa4p can also affect the survival of stress. Yeast cells carrying *SSA1* as the only gene of the *SSA* family are sensitive to a variety of treatments, but overexpression of *SSA4* can drastically increase survival rates when these cells are stressed. Surprisingly, when compared with cells synthesizing Ssa1p only, expression of *SSA4(1-236)-GFP* also somewhat improved stress survival, but in contrast to the expression of *GFP-SSA4(16-642)* the differences were not statistically significant. The fact that GFP-Ssa4p(16-

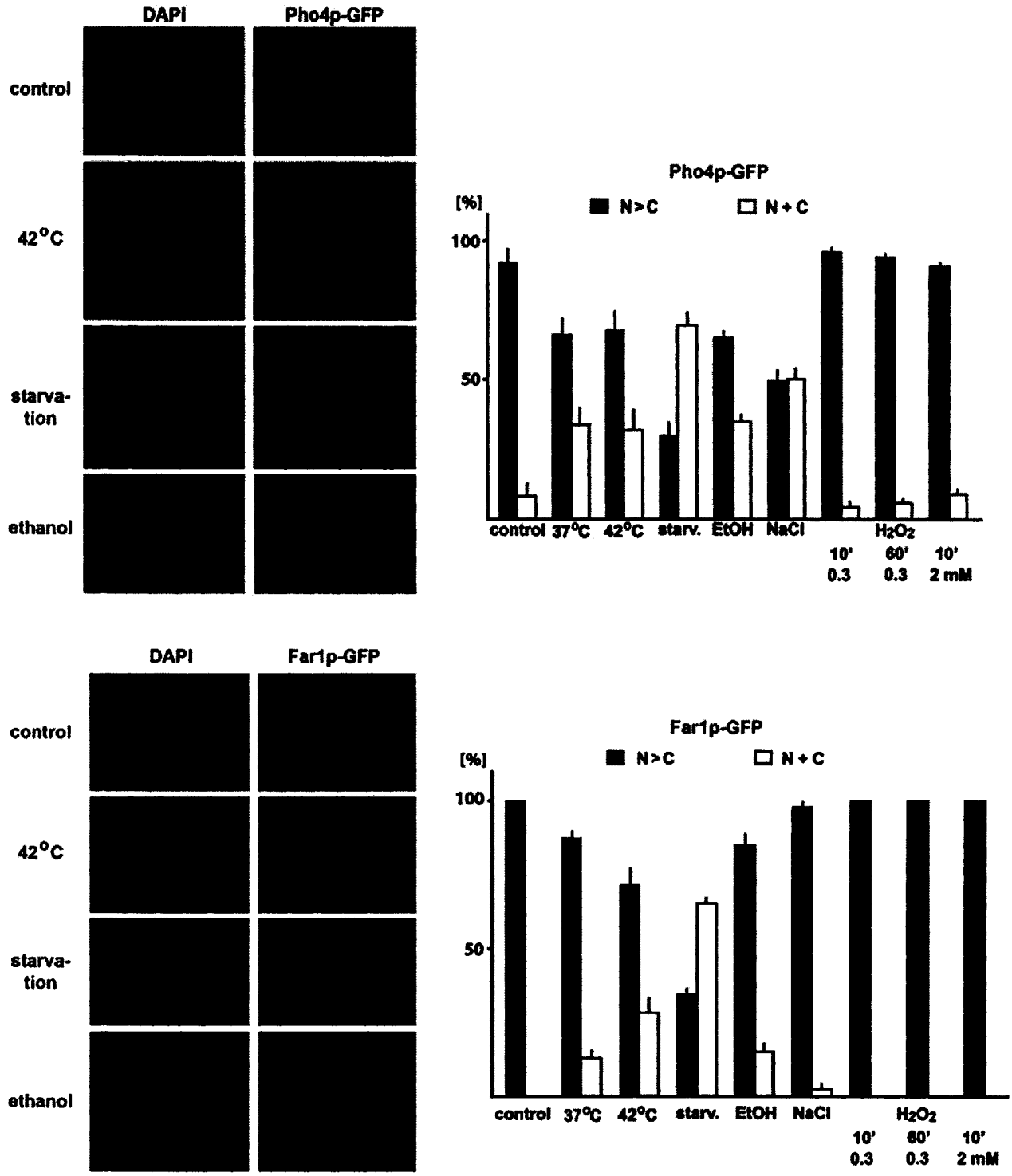

Fig. 9. The localization of Pho4p and Far1p is sensitive to stress. The distribution of Pho4p-GFP and Far1p-GFP was analysed under the same conditions as shown for Fig. 8.

642), but not Ssa4p(1-236)-GFP, improved survival of stressed cells suggests that the C-terminal peptide binding domain participates in the protection against stress-induced damage. These data are consistent with the idea that the chaperone activity of Ssa4p contributes to the increase in stress survival.

Our studies show that nuclear export mediated by Msn5p is crucial for the regulation of Ssa4p distribution

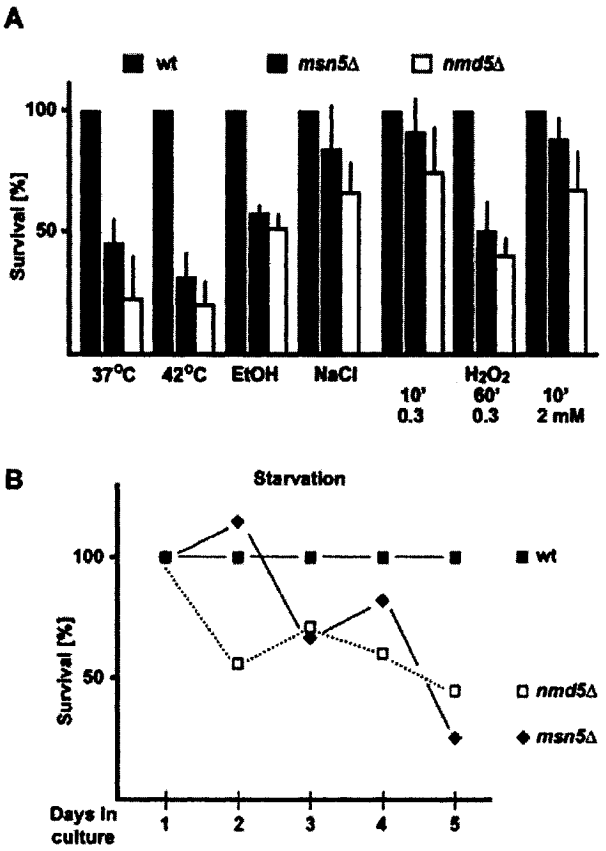

Fig. 10. Mutant strains *msn5Δ* and *nmd5Δ* show increased sensitivity to stress. Wild-type (W303) and mutant strains lacking a functional *MSN5* or *NMD5* gene were stressed (A) or starved (B) as detailed in Fig. 4 and immediately plated out. The colony formation of mutant and wild-type cells was compared. Survival of wild-type cells was defined as 100% for each of the different stresses. Under all stress conditions tested *msn5Δ* and *nmd5Δ* displayed a lower viability than the wild-type strain. Means and standard deviations for three independent experiments are shown.

under non-stress conditions. Moreover, not only the cargo Ssa4p, but also the localization of its carrier Msn5p is controlled by the physiological state of the cell. As such, Msn5p localization is sensitive to stress exposure as well as the composition of the growth medium. In particular, growth on non-fermentable carbon sources, ethanol, heat, starvation or severe oxidative stress induces a redistribution of Msn5p from nuclei to the cytoplasm. To our knowledge, these results are the first studies to report how different stress and growth conditions regulate the localization of a nuclear transporter. Interestingly, the presence of Msn5p in the cytoplasm was found to correlate with the nuclear accumulation of Ssa4p. It is possible that the cytoplasmic localization of Msn5p may limit the trafficking across the nuclear envelope and play a role in the regulation of specific nuclear import and export processes. Moreover, stress conditions that relocate Msn5p to the cytoplasm also inhibit the complex formation between Ssa4p(1-236)-GFP and Msn5p. It is tempting to speculate that Msn5p under these conditions is unable to interact with export cargo. For instance, it is possible that post-translational modifications of carrier and/or cargo in stressed cells prevent the interaction between Msn5p and Ssa4p(1-236)-GFP, and this question will have to be addressed in future studies.

We do not expect that the availability of Msn5p in the nucleus is the limiting factor that controls nucleocytoplasmic trafficking for all of its cargos. For example, the transcription factors Mig1p and Pho4p display a complex transport regulation which requires their modification for Msn5p-dependent translocation to the cytoplasm (Ferrigno *et al.*, 1998; Kaffman *et al.*, 1998; reviewed in Hood and Silver, 1999). Our data for Mig1p show clearly that Msn5p distribution is not the critical reaction for localizing this transcription factor. The relative abundance of a particular cargo and the affinity for the carrier may also play a role in its steady-state distribution. As such, Ssa4p is more than 22 times as abundant when com-

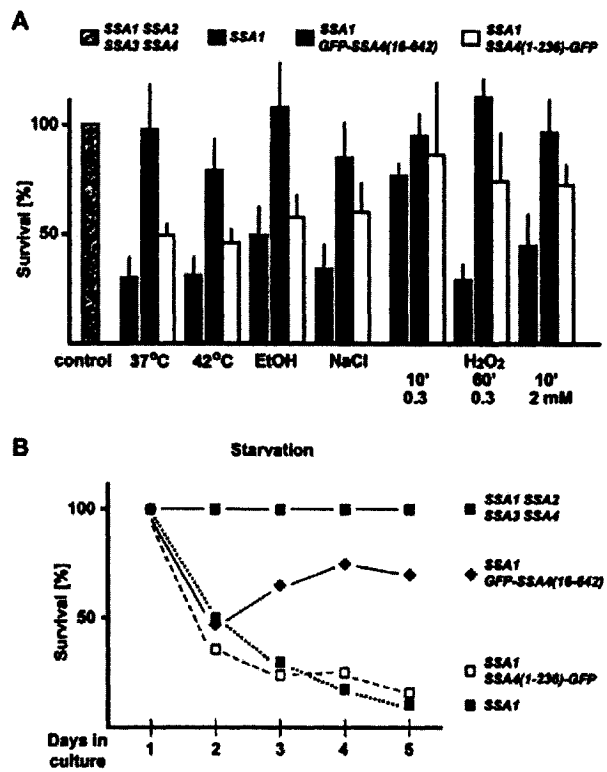

Fig. 11. Ssa4p increases the viability of stressed yeast cells. Yeast cells carrying genes *SSA1 SSA2 SSA3 SSA4* (control), *SSA1*, *SSA1* and *GFP-SSA4(16-642)* or *SSA1* and *SSA4(1-236)-GFP* were incubated under the conditions given in Fig. 4 and plated out immediately. Survival rates for *SSA1 SSA2 SSA3 SSA4* were defined as 100%. Means and standard deviations are depicted for three independent experiments. For all treatments *GFP-SSA4(16-642)* significantly increased the viability of stressed *SSA1* cells ($P < 0.02$, Student's *t*-test).

Table 1. Yeast strains used in this study.

Strain	Genotype	Reference
W303	*Matα ade2 his3 leu2 trp1 ura3*	
msn5Δ	*Matα ade2 his3 leu2 trp1 ura3 can1 msn5::Tn LEU2*	Chughtai et al. (2001)
MSN5-MYC	*ade2 his3 leu2 ura3 trp1 can1 MSN5-MYC*	Alepuz *et al.* (1999)
kap114Δ	*his3 leu2 ura3 trp1 kap114::TRP1*	Chughtai *et al.* (2001)
xpo1-1	*Matα ade2 his3 leu2 ura3 trp1 xpo1::LEU2* (pKW-*HIS3-xpo1-1*)	Stade *et al.* (1997)
XPO1	*Matα ade2 his3 leu2 ura3 trp1 xpo1::LEU2* (pKW-*HIS3-XPO1*)	Stade *et al.* (1997)
XPO1 T539C	*ura3 XPO1/CRM1 T539C*	Neville and Rosbash (1999)
nup82-3	*Matα ade2 his3 leu2 ura3 trp1*	Belanger *et al.* (2004)
NUP1	*Matα his3 leu2 trp1 ura3 nup1::LEU2* (*NUP1-TRP-CEN*)	
nup1-8	*Matα his3 leu2 trp1 ura3 nup1::LEU2* (*nup1-8-TRP-CEN*)	
nsp1-5	*Matα ade2 his3 leu2 trp1 ura3 nsp1::HIS3* (*pSB32-LEU2-nsp1-5*)	Nehrbass *et al.* (1990)
JN55	*his3 leu2 lys2 trp1 ura3 SSA1 SSA2 SSA3 SSA4*	Becker *et al.* (1996)
JN516	*his3 leu2 trp1 ura3 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2 SSA1*	Becker *et al.* (1996)

pared with Mig1p and about five times as abundant as Msn5p (Ghaemmaghami *et al.*, 2003). Moreover, in the case of Mig1p and Pho4p binding to Msn5p is controlled by phosphorylation. Thus, competition between different proteins for binding to the carrier can add another layer of complexity that controls the localization of individual cargos.

In summary, we have shown a correlation between the localization of Msn5p in the cytoplasm and Ssa4p in the nucleus. Whenever elevated levels of Msn5p are present in the cytoplasm increased concentrations of Ssa4p are detected in the nucleus. On the basis of this observation, it is tempting to speculate that the steady-state distribution of Msn5p may contribute to the export efficiency of some cargos. It is conceivable that regulating the levels of Msn5p in the nuclear or cytoplasmic compartment could provide an additional tool to adjust nuclear trafficking of selected cargos to the needs dictated by the physiological state of the cell.

Experimental procedures

Strains, plasmids and growth conditions

Yeast strains are shown in Table 1. Plasmids encoding Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) have been described previously (Quan *et al.*, 2004). For the experiments shown in Fig. 1, DNA encoding *GAL1* and *SSA4(1-236)-GFP* was cloned into a centromeric plasmid carrying *HIS3* as a selectable marker. Wild-type *MSN5* was introduced with plasmid pEL335 (Alepuz *et al.*, 1999), which is referred to as p*MSN5*. Alternatively, wild-type *MSN5* was provided on a centromeric plasmid (Blondel *et al.*, 1999; Fig. S2). Cells were grown in rich (1% yeast extract, 2% peptone) or drop out medium containing 2% glucose, 2% galactose, 3% glycerol or 2% ethanol as a carbon source. Yeast transformation followed standard procedures; transformants were selected and grown on complete minimal medium lacking amino acids or nutrients used for selection.

Localization of GFP-fusion proteins and indirect immunofluorescence

Fusion proteins containing the GFP-tag were localized as described previously (Quan *et al.*, 2004). In brief, samples were analysed with a Nikon Optiphot at 1000× magnification. To avoid the non-linear range of fluorescence signals, cells highly overexpressing GFP or a GFP-tagged fusion protein were excluded from further analyses. Myc-tagged Msn5p inserted into the chromosome (Alepuz *et al.*, 1999) was detected by indirect immunofluorescence with mab9E10 (Santa Cruz Biotechn., sc-40) essentially as in Gao *et al.* (2003). The localization of proteins was monitored by visual inspection of the specimens. At least 100 cells were scored in each of at least three independent experiments. The distribution of fluorescence was scored as described in Quan *et al.* (2004). N >> C denotes fluorescence can be detected in nuclei only, N > C, nuclear accumulation with weak signals detected in the cytoplasm; N + C, equal fluorescence signals are observed in nucleus and cytoplasm. Within every set of experiments, the same exposure time was used for all images. Negatives were scanned and processed with Adobe Photoshop 8.0. Alternatively, images were taken with a Zeiss LSM 510 and processed in Adobe Photoshop 8.0.

Purification of tagged proteins from bacteria

GST- and His6-tagged proteins were synthesized in *Escherichia coli*. GST and GST-Gsp1p have been described previously (Quan *et al.*, 2004). A plasmid construct encoding GST-Msn5p was provided by G. Schlenstedt, His6-tagged versions of Rna1p, Ssa4p(1-236)-GFP and Ssa4(16-642) were generated by us using conventional methods. Proteins were purified under native conditions following standard procedures using glutathione Sepharose 4B (Amersham Biosciences, Baie d'Urfé, QC) or Ni-NTA agarose (Qiagen, Mississauga, ON).

Purification of Msn5p containing complexes

Protein complexes containing Myc-tagged Msn5p were isolated with Myc-specific antibodies under native conditions. In

brief, cells synthesizing Myc-Msn5p and GFP-Ssa4p(16-642), Ssa4p(1-236)-GFP or GFP were grown overnight in selective medium containing 2% galactose to induce the synthesis of GFP-Ssa4p(16-642), Ssa4p(1-236)-GFP or GFP. Cells were grown to an OD_{600} of 1–1.3 and harvested by centrifugation. Sediments were stored at –70°C until use. All subsequent steps for lysis and immunoprecipitation were carried out at 4°C. The equivalent of 5 OD_{600} units was lysed with glass beads in buffer A [20 mM Pipes/KOH, 250 mM sorbitol, 150 mM potassium acetate, 5 mM magnesium acetate, pH 7.5, 1% Triton X-100, 0.5 mM DTT, 1 mM PMSF, 5 mM benzamidine, and a protease inhibitor cocktail (antipain, aprotinin, chymostatin, leupeptin and pepstatin; each at 1 μg ml^{-1})]. Extracts were centrifuged for 5 min at 13 000 rpm (microfuge), and 1 ml of the supernatant was incubated with gentle agitation with 1 μg anti-Myc antibodies. After 2 h, 10 μl of protein G-Sepharose (Amersham Biotech.) equilibrated in buffer A was added and samples were incubated overnight. The resin was collected by centrifugation and washed three times with 250 μl buffer A. Material bound to the resin was released by boiling in gel sample buffer (10 min, 95°C) and supernatants (1 min, 13 000 rpm, microfuge) were analysed by Western blotting with antibodies against GFP (Quan *et al.*, 2004). To test the effect of the Gsp1p-GAP, His6-tagged Rna1p was purified from bacteria and added to 5 μM before the addition of anti-Myc antibodies.

Western blotting and quantization of ECL signals

Western blot analysis with antibodies against GFP, the His6- or Myc-epitope and quantization of signals was carried out as described (Quan *et al.*, 2004). In brief, 'Spot Density Tools' software was used to measure ECL signals as recommended by Alpha Innotech Corporation (San Leandro, CA). The density was determined for each area of interest and corrected for background. All of the samples measured for a particular experiment were present on the same filter. Films were exposed for different times to ensure that the brightest signal was below the saturation level of the film. The quantification was carried out for at least three independent experiments.

In vitro *complex formation between Ssa4p and Msn5p*

Ni-NTA resin (Qiagen) was equilibrated with PBS/KMT buffer (25 mM sodium phosphate, pH 7.3, 150 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 0.1% Tween 20, 1 mg ml^{-1} BSA and a cocktail of protease inhibitors). One hundred and twenty microlitres of Ni-NTA resin was loaded with His6-Ssa4p(16-642) or Ssa4p(1-236)-GFP-His6 (each at 40 nM) for 1 h at 4°C and washed three times with 1 ml PBS/KMT buffer. The resin was divided into four identical aliquots and incubated in a final volume of 200 μl, containing 1 mM GTP and 40 nM GST-Msn5p, 200 nM GST-Gsp1pQ71L, 40 nM GST, alone or in combination (see Fig. 4). Samples were incubated for 1 h at 4°C with gentle agitation and beads were washed three times in 1 ml PBS/KMT. Bound proteins were eluted in gel loading buffer (10 min, 95°C) and further analysed by SDS-PAGE and Western blotting with antibodies against GFP and GST.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. A. Nuclear export of Ssa4p(1-236)-GFP or GFP-Ssa4p(16-642) does not require the carrier Xpo1p. Yeast cells carrying the LMB-sensitive *CRM1 T539C* allele were grown at room temperature and exposed to LMB for the times indicated. For comparison, the fluorescent shuttling protein NES-NLS-GFP2 was analysed. NES-NLS-GFP2 accumulates rapidly in nuclei upon addition of LMB, whereas Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) fail to concentrate in nuclei under the same conditions. The location of Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) was quantified in untreated controls, cells incubated with the solvent only or treated with 100 ng ml^{-1} LMB. The percentage of cells that accumulate Ssa4p(1-236)-GFP or GFP-Ssa4p(16-642) in nuclei (N > C) was determined for three independent experiments; at least 100 cells were scored for each experiment. Means and standard deviation are shown.

B. Xpo1p is not essential for Ssa4p nuclear export. Mutant *xpo1-1* cells were incubated at room temperature or heat-treated for 15 min or 30 min at 37°C to inactivate

the temperature-sensitive carrier. Ssa4p(1-236)-GFP and GFP-Ssa4p(16-642) were localized by fluorescence microscopy. Quantification was carried out as described for Fig. 1.

Fig. S2. *MSN5* provided on a centromeric plasmid rescues ethanol-induced nuclear accumulation of Ssa4p(1-236)-GFP in *msn5*Δ cells. Wild-type *MSN5* was introduced on a centromeric plasmid and Ssa4p(1-236)-GFP was localized as in Fig. 1.

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