Starvation promotes nuclear accumulation of the hsp70 Ssa4p in yeast cells

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Running title: Nuclear accumulation of hsp70s in starving yeast cells

Keywords: nucleus, hsp70, starvation, nuclear transport, importins, Nmd5p.

Abbreviations: β-galactosidase, Escherichia coli β-galactosidase; cNLS, classical nuclear localization sequence; DAPI, 4',6-diamidino-2-phenylindole; DEM, diethyl maleate; GFP, Aequorea victoria green fluorescent protein; NES, nuclear export sequence; NLS, nuclear localization sequence; NPC, nuclear pore complex; Star, protein sequence inducing nuclear targeting upon starvation.
Abstract

Nuclear import of proteins which are too large to passively enter the nucleus requires soluble factors, energy, and a nuclear localization signal (NLS). Nuclear protein transport can be regulated, and different forms of stress affect nucleocytoplasmic trafficking. As such, import of proteins containing a classical NLS (cNLS) is inhibited in starving yeast cells. In contrast, the hsp70 Ssa4p concentrates in nuclei upon starvation. Nuclear concentration of Ssa4p in starving cells is reversible, and transfer of stationary phase cells to fresh medium induces Ssa4p nuclear export. This export reaction represents an active process that is sensitive to oxidative stress. In starving cells, the N-terminal domain of Ssa4p mediates Ssa4p nuclear accumulation, and a short hydrophobic sequence, termed Star (for starvation), is sufficient to localize the reporter proteins GFP or β-galactosidase to nuclei. To determine whether nuclear accumulation of Star-β-galactosidase depends on a specific nuclear carrier, we have analyzed its distribution in mutant yeast strains that carry a deletion of a single β-importin gene. With this assay we have identified Nmd5p as a β-importin required to concentrate Star-β-galactosidase in nuclei when cells enter stationary phase.
Introduction

In eukaryotic cells, DNA replication and RNA synthesis take place in the nucleus, whereas protein synthesis occurs in the cytoplasm. Proper communication between these processes depends on the transport of soluble factors between both compartments. Nucleocytoplasmic trafficking requires that proteins cross the nuclear envelope. In order to do so, proteins travel through nuclear pore complexes (NPCs), large specialized structures that span both the inner and outer nuclear membrane. Proteins that are smaller than 40-60 kD can diffuse through NPCs without the requirement of energy. In contrast, macromolecules with a molecular mass larger than 40-60 kD enter the nucleus via active transport (reviewed in 1, 2).

A variety of pathways mediate the nuclear accumulation of proteins too large to diffuse into the nucleus (reviewed in 1, 2). In most cases, nuclear trafficking depends on specific carrier molecules, termed importins. In the yeast *Saccharomyces cerevisiae* 14 members of the β-importin family have been identified (reviewed in 3), three of which are essential for cell viability. These carriers are involved in nuclear export or import of proteins and RNA. At present, classical nuclear protein transport is the pathway understood best. This transport route requires an adaptor protein, α-importin (Sr1p in yeast) which links the nuclear cargo to β-importin (Rsl1p in yeast). Like other forms of nuclear transport, classical nuclear protein import requires energy, soluble transport factors, and nuclear localization sequences (NLSs), specialized signals that target proteins into the nucleus. In classical nuclear transport, these signals can be of two types: monopartite or bipartite. Monopartite signals are simple stretches of basic amino acids, whereas bipartite NLSs contain two clusters of basic amino acids separated by a spacer region (reviewed in 4).
In addition to the well-characterized classical nuclear import route, a variety of non-classical pathways have been described which depend on other members of the β-importin family of carriers (3). Frequently, non-classical transport pathways are mediated by NLSs distinct from the simple or bipartite type, and such non-classical NLSs do not share a consensus sequence. Examples of proteins containing a non-classical NLS include the mammalian protein hnRNPA1 and the yeast protein Npl3p (1, 2). In general, proteins bearing a non-classical NLS associate directly with a specific β-importin for targeting to the nucleus, without the requirement of an adaptor. Thus, β-importins have been shown to play a role in nuclear import of ribosomal proteins, transcription factors or RNA-binding proteins (3). Of particular interest are β-importins involved in trafficking of proteins that regulate the response to stress or changes in nutrient availability. For instance, Nmd5p mediates nuclear import of the MAP kinase Hog1p upon exposure to osmotic stress, and nuclear import is triggered by Hog1p phosphorylation (5). Another example of regulated nucleocytoplasmic trafficking is Mig1p, a transcription factor promoting glucose-mediated repression of several genes (6). Removal of glucose from the growth medium induces Mig1p phosphorylation via Snf1p. Phosphorylated Mig1p is then recognized by the β-importin Msn5p and subsequently exported to the cytoplasm (6).

During logarithmic growth, cells deplete their medium for nutrients and enter stationary phase. Under these conditions, a variety of genes modify their expression levels. Nutrient depletion represents a specific form of stress that inhibits classical nuclear transport and can also affect non-classical transport pathways (7). Moreover, various proteins regulating the response to changing nutrient concentrations, such as Mig1p, may alter their distribution between nucleus and cytoplasm (reviewed in 8).
Different forms of stress alter the cellular physiology and may damage cells. To survive and recover from stress-induced injury, cells need to activate specialized survival and repair mechanisms. In particular, heat shock proteins of the hsp70/hsc70 family play an essential role in these processes. Hsp70/hsc70s shuttle between nucleus and cytoplasm (9) and accumulate in nuclei upon exposure to heat stress. Unlike heat shock, however, the effect of starvation on hsp70 localization has not been studied in detail.

The yeast *Saccharomyces cerevisiae* contains six members of the cytosolic hsp70 family. Cytosolic hsp70s are divided into two subfamilies: four SSA encoded gene products (Ssa1p to Ssa4p), and two SSB gene products (Ssb1p and Ssb2p) (reviewed in 10). The SSA4 gene is particularly interesting as its expression is highly induced upon stress (10) and upregulated during diauxic shift. Proteins of the SSA family are located in the cytoplasm and the nucleus, as they shuttle between both compartments under normal conditions. Unlike gene products of the SSA group, Ssb1p and Ssb2p are located predominantly in the cytoplasm. Ssb1p is prevented from nuclear accumulation due to the presence of a nuclear export signal (NES) in its N-terminal domain (11).

It has not been analyzed previously whether nutrient depletion affects the localization of hsp70s. We have addressed this problem by generating fusions containing Ssa4p and GFP (*Aequorea victoria* green fluorescent protein). We now show that upon starvation GFP-fusion proteins carrying Ssa4p or its N-terminal domain accumulate in nuclei. We have identified a short hydrophobic segment in this N-terminal portion, referred to as the Star sequence, which is sufficient to direct non-nuclear reporter proteins to nuclei when cells are entering stationary
phase. Moreover, we demonstrate that the β-importin Nmd5p is required to import a fusion between the Star sequence and β-galactosidase into nuclei of early stationary phase cells.
Experimental Procedures

Yeast Strains and Growth of Cells

Yeast strain RS453 (ade2 ADE3 leu2 ura3 trp1 his3), provided by V. Doye (Paris) was used as wild type strain. Mutant yeast strains were provided by D. Mangroo (Guelph) G. Schlenstedt (Homburg). Upon transformation cells were grown in synthetic complete medium lacking uracil or leucine ("dropout medium"), depending on the selectable marker introduced by the plasmid. Cells were kept at room temperature unless indicated otherwise. Expression of genes controlled by the GAL1 or GALS promoter was induced with 2% galactose as a carbon source.

For data shown in Figure 2, cells were transferred for 6 hours into fresh glucose-containing medium. The following inhibitors were added as indicated in the figure: 2 mM hydrogen peroxide (American Chemicals, Montreal), 2 mM diethyl maleate (DEM, Sigma, Oakville, Ontario) or 100 µg/ml cycloheximide (Sigma).

Plasmids and Transformation of Yeast Cells

All plasmid constructions were carried out in E. coli strain XL1-Blue. The correctness of constructs was verified by sequencing with the sequenase 2.0 kit (USB, Cleveland, OH). To generate fusions between GFP and SSA4, the GFPmut1-allele was used. GFPmut1 encodes a protein with approximately 35-times the fluorescence intensity of the wild type (12). Plasmid pGAD-GFP encodes NLS-GFP, a fusion protein that carries the classical SV40-NLS (13). pGAD-GFP was kindly provided by Dr. D. Goldfarb (Rochester). GFP-Ssa4p(16-642): A NotI-linker (12mer, New England Biolabs, Mississauga, ON) was inserted into the unique NlaIII-site of SSA4, thereby generating plasmid p930. Plasmid p930 was cut with NotI, and the GFP-coding sequence was fused in frame to codon 16 of SSA4, the fusion gene encodes GFP-
Ssa4p(16-642). For expression in yeast, the centromeric plasmid, which carries the \textit{URA3} selectable marker, was used as a vector. Expression of the gene fusions inserted into this plasmid is regulated by the \textit{GAL1} promoter. Generation of GFP-Star: GFP containing a NotI-linker at the 3’-end (14) was fused to oligonucleotides encoding amino acid residues Ile162 to Ile171 of Ssa4p, followed by an UAG stop codon. To generate fusions between the Star sequence and β-galactosidase, oligonucleotides encoding methionine followed by the Star sequence were fused to the 5’-end of the β-galactosidase gene. To obtain GFP-β-galactosidase, EGFP (Clontech, Palo Alto, CA) was fused in frame to the 5’-end of β-galactosidase. For expression in yeast, the Star-β-galactosidase gene was cloned into centromeric plasmids with either \textit{LEU2} or \textit{URA3} as a selectable marker.

Yeast cells were transformed as described previously (14) and selected on dropout plates containing 2% glucose.

\textit{Fluorescence Microscopy}

GFP-containing reporter proteins were located by fluorescence microscopy as previously described (14). Fusion proteins carrying β-galactosidase were located with monoclonal antibodies as detailed in ref. 15. To detect GFP-Star and Star-β-galactosidase simultaneously, cells were grown in synthetic complete medium lacking both uracil and leucine and containing 2% galactose. Cells were fixed, permeabilized and incubated overnight with monoclonal antibodies against β-galactosidase (diluted 1:500) and polyclonal antibodies against GFP (1:50, CLONTECH, Palo Alto). Primary antibodies were detected with Cy3-conjugated affinity purified antibodies against mouse IgG and FITC-conjugated affinity purified antibodies to rabbit IgG (Jackson ImmunoResearch, West Grove, PA). DNA was visualized with 4’,6-
diamidino-2-phenylindole (DAPI) and slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA).
Results

*GFP-Ssa4p fusion proteins accumulate in nuclei of starving cells* - Fusions of GFP and members of the SSA family of proteins are proper models to study their targeting within the cell (11). As described below, we have used proteins containing GFP and distinct segments of the cytoplasmic hsp70 Ssa4p for our studies. In logarithmically growing cells, the fusion protein GFP-Ssa4p(16-642), which contains amino acid residues 16 to 642 of the authentic sequence of Ssa4p (642 amino acid residues), was both nuclear and cytoplasmic (Figure 1, A, B). By contrast, this protein accumulated in nuclei of stationary phase cells (Figure 1, C, D). GFP-Ssa4p(16-642) could exit the nucleus when cells were provided with fresh medium containing glucose (Figure 1, E-F). This appearance in the cytoplasm did not reflect *de novo* gene expression, since glucose inhibits transcription from the *GAL1* promoter. Export of GFP-Ssa4p(16-642) from the nucleus was independent of the carbon source in the growth medium as the same result was obtained for cells incubated with non-fermentable substrates such as glycerol (Figure 1, G, H). Furthermore, nuclear export of GFP-Ssa4p(16-642) was an active process which was inhibited if cells were kept at 4°C (Figure 1, I, J). For comparison, we have analyzed the localization of the GFP-tag and NLS-GFP. GFP distributed throughout the nucleus and cytoplasm under different growth conditions (Figure 1, K-N). NLS-GFP, a substrate for classical nuclear transport, concentrated in nuclei of control cells but equilibrated between nucleus and cytoplasm in starving cells (Figure 1, O-R, and 7).

*Nuclear export of GFP-Ssa4p(16-642) is sensitive to oxidative stress* - Nuclear accumulation of GFP-Ssa4p(16-642) in starving cells was reversible and transfer to fresh medium promoted its export into the cytoplasm. Nucleocytoplasmic trafficking, and in particular, nuclear export
of several proteins, is sensitive to oxidants (16, 17; reviewed in 8). When stationary phase cells were transferred to fresh medium containing glucose, both hydrogen peroxide and diethyl maleate strongly inhibited nuclear export of the fusion protein (Figure 2, C-F). In contrast, the protein synthesis inhibitor cycloheximide had no effect on nuclear export, and GFP-Ssa4p(16-642) appeared in the cytoplasm, as was observed in control cells (compare A, B with G, H in Figure 2).

The N-terminal domain of Ssa4p targets GFP to nuclei of starving cells - To define regions of Ssa4p that are sufficient for nuclear accumulation when cells enter stationary phase, a fusion of the authentic 236 amino acid residues of Ssa4p to GFP was generated. This fusion protein, referred to as Ssa4p(1-236)-GFP, lacks the bipartite NLS present in Ssa4p. In logarithmically growing cells, Ssa4p(1-236) was found in the cytoplasm and the nucleus (Figure 3, A, B), similar to GFP-Ssa4p(16-642). Moreover, Ssa4p(1-236)-GFP concentrated in nuclei of starving cells (Figure 3, C, D), demonstrating that the N-terminal segment of Ssa4p was sufficient to promote nuclear accumulation in stationary phase cells. As a control for a non-nuclear protein, the distribution of GFP-β-galactosidase was also monitored. This protein was cytoplasmic, with nuclei and vacuoles being excluded (Figure 3, I, J). It should be noted that nuclear accumulation of GFP-Ssa4p(16-642), Ssa4p(1-236), GFP-Star or Star-β-galactosidase (see below) was never complete, and a portion of the protein was always detected in the cytoplasm. Nuclear accumulation was also transient and preferentially detected in early stationary phase cells.
A short hydrophobic segment of the N-terminal Ssa4p domain is sufficient for nuclear accumulation in starving cells - Amino acid residues 162 to 171 of Ssa4p are mostly hydrophobic. The sequence ile-ala-gly-leu-asn-val-leu-arg-ile-ile (referred to as Star sequence, for "starvation") fits the consensus sequence proposed for hydrophobic nuclear export signals (16). However, when fused to GFP, we did not detect nuclear exclusion (data not shown), but GFP-Star equilibrated between nucleus and cytoplasm in exponentially growing cells (Figure 3, E, F). Importantly, GFP-Star concentrated in nuclei of starving cells (Figure 3, G, H).

Three different mechanisms could explain nuclear accumulation of GFP-Star in nutrient-depleted cells. In one scenario, due to its small size, GFP-Star diffuses into nuclei and may be retained when cells are starving. This would involve an interaction with nuclear proteins which provide anchors that prevent GFP-Star from diffusing into the cytoplasm. Alternatively, the Star sequence could function as an NLS which is active only under certain physiological conditions. Finally, a combination of nuclear retention and nuclear import may concentrate in nuclei reporter proteins containing the Star sequence. To begin to address these questions, we have generated the fusion protein Star-β-galactosidase. This fusion protein is too large to translocate across the nuclear envelope by passive diffusion; therefore its nuclear localization would require transport into nuclei by an active process. As shown in Figure 4, Star-β-galactosidase was able to accumulate in nuclei of stationary phase cells, supporting the idea that the Star sequence can function as an NLS. To compare the distribution of GFP-Star and Star-β-galactosidase under identical conditions, both proteins were synthesized in the same cell and localized in logarithmically growing as well as stationary phase cells (Figure 4, A to F). GFP-Star and Star-β-galactosidase colocalized and concentrated in nuclei when cells were entering stationary phase (Figure 4, D to F). Upon prolonged incubation of cells for several
days in nutrient-depleted medium the levels of GFP-Star and Star-β-galactosidase were reduced, and we no longer detected a concentration of the reporter proteins in nuclei (data not shown). This result most likely reflects protein degradation in starving cells.

_GFP-Star accumulates in nuclei of yeast strains carrying mutations in β-importin genes -_  
We have tested the nuclear accumulation of GFP-Star in yeast strains carrying a deletion in one of the β-importin genes. With the experiments shown in Figure 5 and summarized in Table 1, we followed the localization of GFP-Star in cells during logarithmic growth and in stationary phase. All of the mutants tested equilibrated GFP-Star between nucleus and cytoplasm when cells were growing logarithmically. However, GFP-Star accumulated in nuclei when cultures were in early stationary phase. These results are consistent with the interpretation that the concentration of the small protein GFP-Star in nuclei does not depend on one particular β-importin and may be achieved by nuclear retention only.
Nmd5p is required to accumulate Star-β-galactosidase in nuclei of early stationary phase cells

To determine whether the deletion of a β-importin gene affects active nuclear import mediated by the Star-sequence, we monitored the distribution of Star-β-galactosidase during logarithmic and stationary phases of the culture. As shown in Figure 6 and summarized in Table 1, most of the deletion mutants were able to accumulate Star-β-galactosidase in nuclei, supporting the idea that they are not required for nuclear import mediated by the Star-sequence. However, cells lacking a functional NMD5 gene failed to concentrate Star-β-galactosidase in nuclei of early stationary phase cells (Figure 6, Table 1), indicating that Nmd5p plays a role in these transport reactions.

As a possible complication of our experiments, overexpression of Star-β-galactosidase may result in high levels of cytoplasmic protein, which could mask its nuclear accumulation. To obtain lower levels of gene expression, we cloned the Star-β-galactosidase gene into vectors carrying the GALS promoter (18). However, when we localized Star-β-galactosidase in nmd5::TRP1 cells transformed with these plasmids, the reporter protein did not concentrate in nuclei when cells entered stationary phase (data not shown). These results further support the idea that Nmd5p is involved in nuclear import mediated by the Star sequence.
Discussion

Changes in physiological conditions require cells to adapt, and in response to various types of stress proteins may alter their intracellular localization. In particular, trafficking between nucleus and cytoplasm is affected in cells exposed to oxidative or osmotic stress (reviewed in 8). Furthermore, changes in nutrient concentration have been shown to alter protein localization (6, 19, 20). For instance, the distribution of regulatory and catalytic subunits of protein kinase A in *Saccharomyces cerevisiae* depends on the carbon source (20). Likewise, the distribution of hepatic glucokinase is altered in response to metabolic changes (19). The effect of nutrient-depletion on nucleocytoplasmic transport of heat shock proteins, however, has not been defined previously.

Proteins of the hsp70 family are key components in the stress response, a process that is conserved among eukaryotes. We have now analyzed the effect of starvation on Ssa4p localization in budding yeast. To this end, distinct segments of Ssa4p were fused to the non-nuclear reporter proteins GFP or β-galactosidase. Both GFP-Ssa4p(16-642) and GFP-Ssa4p(1-236) concentrated in nuclei of starving cells during early stationary phase, indicating that the N-terminal domain of Ssa4p is sufficient for nuclear accumulation. As GFP-Ssa4p(1-236) is lacking the bipartite NLS present in authentic Ssa4p, a cNLS is not required to concentrate the protein in nuclei of starving cells. Within the N-terminal domain of Ssa4p we have identified a short stretch of ten mostly hydrophobic amino acid residues, termed Star (for starvation), which is sufficient to promote nuclear concentration in nutrient-depleted cells. Thus, both GFP-Star as well as Star-β-galactosidase accumulate in nuclei of early stationary phase cells. As the sequence context of Star is different in both fusion proteins, it rules out the possibility that an
artificial nuclear targeting sequence was generated by linking the Star sequence to a reporter protein.

Proteins that exceed the diffusion radius of the NPC, like Star-β-galactosidase, must enter the nucleus by active transport, whereas small molecules can diffuse across NPCs. We have demonstrated that Star-β-galactosidase concentrated in nuclei of starving cells, whereas NLS-GFP, a substrate for classical nuclear protein import, failed to accumulate. This suggests differences in the requirements for nuclear accumulation and the cellular apparatus mediating Star-β-galactosidase and cNLS-dependent import. Our hypothesis is further supported by analyses of mutant yeast strains that carry a deletion of one of the β-importin genes, as we have identified Nmd5p as a β-importin that plays an essential role in nuclear accumulation of Star-β-galactosidase when cells enter stationary phase. By contrast, NLS-GFP is believed to be imported into nuclei by the classical nuclear import pathway, requiring α-importin and the β-importin Kap95p, and this pathway is inactivated in starving cells. Moreover, nuclear accumulation in starving cells is not a general feature of proteins carrying a non-classical NLS. For instance, the yeast protein Npl3p contains a non-classical NLS but fails to concentrate in nuclei of starving cells (7). We therefore propose that the Star sequence functions as a special non-classical NLS in starving cells.

*NMD5* is a non-essential gene that encodes a nuclear carrier which imports the MAP kinase Hog1p and the transcription elongation factor TFIIS into nuclei (5, 21). We have now identified the Star sequence as a signal that mediates nuclear transport via Nmd5p. Nuclear import via Nmd5p could occur by direct binding of a Star-containing protein to Nmd5p or by a piggy-back mechanism that involves a linker molecule between Nmd5p and the Star sequence. At present, targeting signals recognized by Nmd5p are not defined, and future experiments will
have to determine whether Nmd5p and the Star sequence interact directly. It should be noted that we did not detect nuclear exclusion of Star-β-galactosidase in cells carrying a deletion of NMD5. This might indicate that low amounts of Star-β-galactosidase enter the nucleus by an Nmd5p-independent mechanism.

Like Star-β-galactosidase, GFP-Star may accumulate in nuclei by a special nuclear import pathway that is activated in starving cells (see model, Figure 7). However, GFP-Star nuclear accumulation did not depend on any of the β-importins we have tested. Specifically, cells lacking a functional NMD5 gene concentrated GFP-Star in nuclei when entering stationary phase. The simplest explanation of these results is that nuclear accumulation of GFP-Star is achieved by nuclear retention. Due to its small size, GFP-Star diffuses in and out of the nucleus. Once translocated across the nuclear envelope, the Star-sequence may bind to anchors which retain GFP-Star in nuclei (Figure 7). On the basis of our results for Star-β-galactosidase and GFP-Star we propose that the Star sequence has two functions: first it supports active nuclear import, a reaction that depends on Nmd5p. Second, the Star sequence associates with nuclear anchors that prevent its exit from the nucleus, a reaction independent of Nmd5p. Both signal functions, i.e., import and retention, seem to be activated when cells enter stationary phase.

Nuclear accumulation of GFP-Ssa4p(16-642) is reversible, indicating that starving cells, when provided with fresh nutrients, export the fusion protein into the cytoplasm. As GFP-Ssa4p(16-642) is too large to exit the nucleus by diffusion, nuclear export has to be mediated by an active process. This is in line with our observation that GFP-Ssa4p(16-642) remains nuclear if cells are transferred to fresh medium but incubated at 4°C. Moreover, nuclear export
of GFP-Ssa4p(16-642) is sensitive to oxidants which are known to inhibit other nuclear export processes (16).

Our data allow us to propose the following model for protein localization mediated by the Star sequence (Figure 7): In logarithmically growing cells a Star-containing protein can be found in the nucleus and in the cytoplasm. The protein accumulates in nuclei of starving cells, a process that is mediated by Nmd5p-dependent nuclear import, with the Star sequence functioning as a special non-classical NLS. Furthermore, nuclear retention promoted by the Star sequence prevents exit into the cytoplasm. At present, we can only speculate about the role of Ssa4p nuclear concentration in early stationary phase. Like nuclear accumulation of hsp70s upon heat-shock, concentration of Ssa4p in nuclei may help reduce the denaturation of proteins in energy-depleted cells. Once provided with fresh nutrients, Ssa4p is released from nuclear anchors and may participate in refolding of nuclear proteins. Ultimately, Ssa4p nuclear export and shuttling will localize the protein to both nucleus and cytoplasm as observed in logarithmically growing cells.
Acknowledgements

We thank V. Doye (Paris), D. Goldfarb (Rochester) and G. Schlenstedt (Homburg) for providing us with strains and the plasmid encoding NLS-GFP. We are grateful to A. Chu (Montreal) for critical reading of the manuscript. US was supported by grants from MRC and NSERC, Canada. US was a scholar of MRC Canada and a chercheur boursier of FRSQ.

References


Figure Legends

Figure 1. Localization of GFP-Ssa4p(16-642), GFP and NLS-GFP in starving cells. Yeast cells synthesizing GFP-Ssa4p(16-642) (A to J), GFP (K to N) or NLS-GFP (O to R) were grown in medium containing galactose at 30°C, either overnight (control, A, B, K, L, O, P) or for two days (stationary, C, D, M, N, Q, R). Stationary phase cells were transferred to fresh medium supplemented with glucose (E, F, I, J) or glycerol (panels G, H). After 6 hours growth in glucose-containing medium (E, F) or overnight growth on glycerol (panels G, H), GFP-Ssa4p(16-642) was localized. For comparison, cells were kept overnight in medium with glucose at 4°C (I, J). DAPI-staining of DNA (A, C, E, G, I, K, M, O, Q) and green fluorescence of reporter proteins (B, D, F, H, J, L, N, P, R) are shown.

Figure 2: Nuclear export of GFP-Ssa4p(16-642) is sensitive to oxidative stress. Stationary phase cells were transferred to fresh glucose-containing medium in the presence 2 mM hydrogen peroxide (C, D), 2 mM DEM (E, F) or 100 μg/ml cycloheximide (G, H). Controls were transferred to fresh medium without any addition (A, B). DNA was stained with DAPI (A, C, E, G) and fusion proteins were located by green fluorescence (B, D, F, H).

Figure 3: Ssa4p(1-236)-GFP and GFP-Star accumulate in nuclei of starving cells. Ssa4p(1-236)-GFP, GFP-Ssa4p(162-171), termed GFP-Star, and GFP-β-galactosidase were localized in exponentially growing cells (A, B, E, F, I, J) and in stationary phase cells (C, D, G, H). DNA (A, C, E, G, I), Ssa4p(1-236)-GFP (B, D), GFP-Star (F, H) and GFP-β-galactosidase (J) were located by fluorescence microscopy (A to I) or indirect immunofluorescence (J).
Figure 4. **Localization of GFP-Star and Star-β-galactosidase.** Exponentially growing (A, B, C) and starving yeast cells (D, E, F) synthesizing GFP-Star and Star-β-galactosidase (Star-β-gal) were located by indirect immunofluorescence. Staining of the DNA (A, D), GFP-Star (B, E) and Star-β-galactosidase (C, F) are shown.

Figure 5. **Nuclear accumulation of the small protein GFP-Star in β-importin mutants.** Yeast cells carrying a deletion of the β-importin genes *YRB4* (A - D), *KAP114* (E, F) and *NMD5* (G, H) were monitored for the distribution of GFP-Star in logarithmic phase (B) or early stationary phase (D, F, H). For comparison, nuclei were stained with DAPI (A, C, E, G).

Figure 6. **Distribution of Star-β-galactosidase in β-importin mutants.** Star-β-galactosidase (Star-β-gal) was localized in early stationary phase cells in mutant yeast strains lacking functional genes for *YRB4* (A, B), *KAP114* (C, D) or *NMD5* (E, F). Star-β-galactosidase was detected with monoclonal antibodies against β-galactosidase (B, D, F), and nuclei were visualized with DAPI (A, C, E).

Figure 7. **Localization of reporter proteins via the Star sequence.** In early stationary phase cells, Star-β-galactosidase and GFP-Star accumulate in nuclei. Nuclear accumulation of Star-β-galactosidase requires a specialized nuclear import pathway that involves Nmd5p. Nuclear retention, independent of Nmd5p, contributes to the nuclear accumulation of proteins carrying the Star sequence, thus preventing nuclear exit of GFP-Star. See text for details.
Table 1. **Distribution of GFP-Star and Star-β-galactosidase in wild type and mutant cells.**

In wild type and mutant yeast strains inactivated for one of the β-importin genes, GFP-Star and Star-β-galactosidase were localized. Cells were analyzed during logarithmic growth and when entering stationary phase, as indicated. It is shown whether the signal was detected in nuclei (N) or cytoplasm (C). N+C denotes that reporter proteins were detected in nucleus and cytoplasm, nuclear accumulation was seen for N>C. Alternative names of the β-importin genes which have been inactivated are given in parentheses.
Ssa4p(1-236)-GFP, control

C

Ssa4p(1-236)-GFP, stationary

d

GFP-Star, control

E

GFP-Star, stationary

G

GFP-β-gal, control

I

DAPI

GFP

A

B

D

F

H

J
yrb4::TRP1, logarithmic phase

yrb4::TRP1, stationary phase

kap114::TRP1, stationary phase

nmd5::TRP1, stationary phase
**yrb4::TRP1, stationary phase**

(A) DAPI

(B) Star-β-gal

**kap114::TRP1, stationary phase**

(C) DAPI

(D) Star-β-gal

**nmd5::TRP1, stationary phase**

(E) DAPI

(F) Star-β-gal
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Starvation promotes nuclear accumulation of the hsp70 Ssa4p in yeast cells
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J. Biol. Chem. published online March 7, 2001

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