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Transcriptional regulation by inner nuclear membrane proteins

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This thesis is dedicated to my parents Dubravka and Neven Boban
and to my sister Ana.

Work is Love made visible.

And if you can't work with love but only with distaste, it is better that you should leave your work and sit at the gate of the temple and take alms of those who work with joy.

Kahlil Gibran, *The Prophet*

ABSTRACT

All cells sense discrete environmental signals and respond by making appropriate adjustments in patterns of gene expression. To achieve this, signaling pathways translate information received at the cell surface into a transcriptional response. An essential feature of inducible signal transduction pathways is the maintenance of the "off" state of the gene expression when inducing signals are absent. This thesis addresses the mechanisms that ensure the repressed state of SPS-sensor regulated genes in the yeast *Saccharomyces cerevisiae.*

Yeast cells use the plasma membrane localized SPS-sensor to sense extracellular amino acids present in their growth environment. In response to amino acids, the SPS-sensor endoproteolytically activates Stp1 and Stp2, two latent cytoplasmic transcription factors with redundant functions. SPS-sensor mediated processing removes cytoplasmic retention motifs within the N-terminal domains of these factors. The shorter activated forms efficiently target to the nucleus, bind promoters of SPS-sensor regulated genes and induce their transcription. Recessive loss-of-function mutations in *ASI1*, *ASI2* and *ASI3* (amino acid sensor independent) genes bypass the requirement of a functional SPS-sensor and constitutively induce SPS-sensor regulated gene expression. The recessive nature of the *asi* mutations suggested that Asi proteins act to negatively regulate SPS-sensor signaling. Interestingly, all three Asi proteins are integral membrane proteins with multiple membrane-spanning segments.

The goal of this thesis has been to elucidate the function of Asi1, Asi2 and Asi3. Genetic and biochemical tests demonstrate that Asi proteins are *bona fide* constituents of the SPS-sensing pathway. All three Asi proteins localize to the inner nuclear membrane where they function in concert to maintain the latent properties of Stp1 and Stp2 under non-inducing conditions. The data indicate that cytoplasmic retention mechanisms, which prevent nuclear accumulation of latent forms of Stp1 and Stp2, are not completely efficient, and low levels of full-length forms of Stp1 and Stp2 are able to enter the nucleus. In cells lacking either of the Asi proteins, the unprocessed forms of Stp1 and Stp2 that enter the nucleus are able to bind SPS-sensor dependent promoters and inappropriately induce gene expression. Thus, the Asi proteins are required to ensure the repressed state of SPS-sensor signaling in the absence of inducing amino acids by restricting promoter access of full-length Stp1 and Stp2. The nuclear factor Dal81 was found to augment the activation potential of Stp1 and Stp2 by enhancing their efficiency to bind SPS-sensor regulated promoters. Thus, Dal81 greatly enhances the sensitivity of the SPS sensing pathway. Strikingly, in cells lacking Dal81, the negative regulatory activity of Asi1, Asi2 and Asi3 is not required to maintain the "off" state of SPS-sensor regulated gene expression. In summary, this thesis documents the discovery of a novel role of inner nuclear membrane proteins and illuminates an additional and unanticipated layer of transcriptional control in eukaryotic cells.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. **Boban M**, Zargari A, Andréasson C, Heessen S, Thyberg J and Ljungdahl PO.
Asi1 is an inner nuclear membrane protein that restricts promoter access of two latent transcription factors.
J Cell Biol. 2006 Jun 5;173(5):695-707

II. Zargari A*, **Boban M*,** Heessen S, Andréasson C, Thyberg J and Ljungdahl PO.
Inner nuclear membrane proteins Asi1, Asi2 and Asi3 function in concert to maintain the latent properties of transcription factors Stp1 and Stp2.
J Biol Chem. 2007 Jan 5;282(1):594-605
*both authors contributed equally to this work.

III. **Boban M** and Ljungdahl PO.
Loss of inner nuclear membrane protein Asi1 in *Saccharomyces cerevisiae* results in constitutive SPS-sensor regulated gene expression that is Dal81 dependent
Submitted

Additional paper not discussed in this thesis:

Heessen S, Andréasson C, **Boban M** and Ljungdahl PO.
Regulation of the protease Ssy5 in receptor-activated proteolysis of the latent transcription factor Stp1
Submitted

ABBREVIATIONS

AAP	amino acid permease
BAF	barrier-to-autointegration factor
ChIP	chromatin immuno-precipitation
ER	endoplasmic reticulum
GABA	γ-aminobutyric acid
INM	inner nuclear membrane
NES	nuclear export signal
NE	nuclear envelope
NLS	nuclear localization signal
NPC	nuclear pore complex
ONM	outer nuclear membrane
UAS	upstream activating sequence

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PREFACE

In order to adapt to changes in the extracellular surroundings, cells have developed sophisticated signaling pathways that enable a proper response to a particular environmental condition by adjusting the patterns of gene expression. Signals initiated at the cell surface have to be transmitted to the nucleus and give rise to a specific transcriptional response. An important feature of signal transduction is that sensitive systems for inducible gene activation have to be balanced with negative regulatory mechanisms ensuring the repressed state of gene expression in the absence of inducing signals.

Amino acid induced SPS-sensor signaling in the yeast *Saccharomyces cerevisiae* provides an attractive model to study mechanisms of signal transduction. This thesis documents studies aimed at understanding how the repressed state of SPS sensor signaling is ensured in the absence of inducing amino acids. We have found a negative regulatory system comprised of three proteins at the inner membrane of the nuclear envelope, which negatively regulates signaling in the absence of amino acids. Remarkably, these proteins function to maintain the latent behavior of two latent cytoplasmic transcription factors, a finding that illuminates a novel aspect of transcriptional control in eukaryotic cells.

In the following two chapters I will introduce the structure and function of the nuclear envelope and present a review of the literature regarding the role of nuclear envelope in transcriptional regulation. Chapter 3 provides an overview of the amino acid induced SPS sensor signaling pathway in yeast. Finally, the results obtained in my experimental work addressing the role of inner nuclear membrane proteins that ensure the “off” state of SPS sensor signaling are summarized and discussed in Chapter 4.

1. NUCLEAR ENVELOPE

The hallmark of eukaryotic cell is the nucleus, which compartmentalizes chromosomes and physically separates processes such as RNA synthesis, RNA processing and ribosome assembly from the cytoplasm. The nucleus is surrounded by the nuclear envelope (NE). Accumulating evidence suggest that the NE provides more than just a physical barrier separating nucleus from the cytoplasm. For instance, proteins of the NE are thought to play an active role in positioning of nucleus within cells, connecting the nucleoskeleton to cytoskeleton, and gene expression. Perhaps one of the most intriguing roles of the NE is its involvement in the control of gene expression and will be discussed in more detail in the next chapter. The aim of this chapter is to give an overview of the NE structure and composition, and introduce processes occurring at the NE, such as nucleo-cytoplasmic transport and integral membrane protein sorting.

1.1 Nuclear envelope - overview

The nuclear envelope (NE) consists of two nuclear membranes, inner and outer (INM and ONM, respectively). INM and ONM are joined by the pore membranes at the nuclear pores, which allow bidirectional transport of molecules between nucleus and cytoplasm (Figure 1). ONM is contiguous with the endoplasmic reticulum (ER) membrane, which is the site of secretory and membrane protein biosynthesis and thus is often covered with ribosomes. The INM and ONM are separated by a luminal space, also called the periplasmic space, which is continuous with the ER lumen. Although poorly characterized, the periplasmic space likely shares many components with the ER lumen (Prunuske and Ullman, 2006). Consistent with the continuity between ER and ONM, integral membrane proteins of the ONM are also largely found throughout the ER membrane. However, some ONM-specific proteins have been reported to exist (Pan et al., 2000; Starr and Fischer, 2005).

In metazoan cells, the nucleoplasmic face of the INM is lined by intermediate filaments, which are composed of lamin proteins. Lamins associate with many proteins, including proteins of the INM and chromatin. Lamin filaments and lamin-associated proteins together form a meshwork called nuclear lamina (Figure 1). Lamins can also be found in the nuclear interior. Interestingly, the genome of *S. cerevisiae* contains no lamin genes and lamin proteins also appear to be absent from plants (Cohen et al., 2001). Since in metazoan cells lamins play an important role in the mechanical stability of cell nuclei, the lack of recognizable nuclear lamina in organisms like yeast and plants entails questions how this is achieved in a lamin-free

system and whether other non-lamin filamentous structures underlying INM exist.

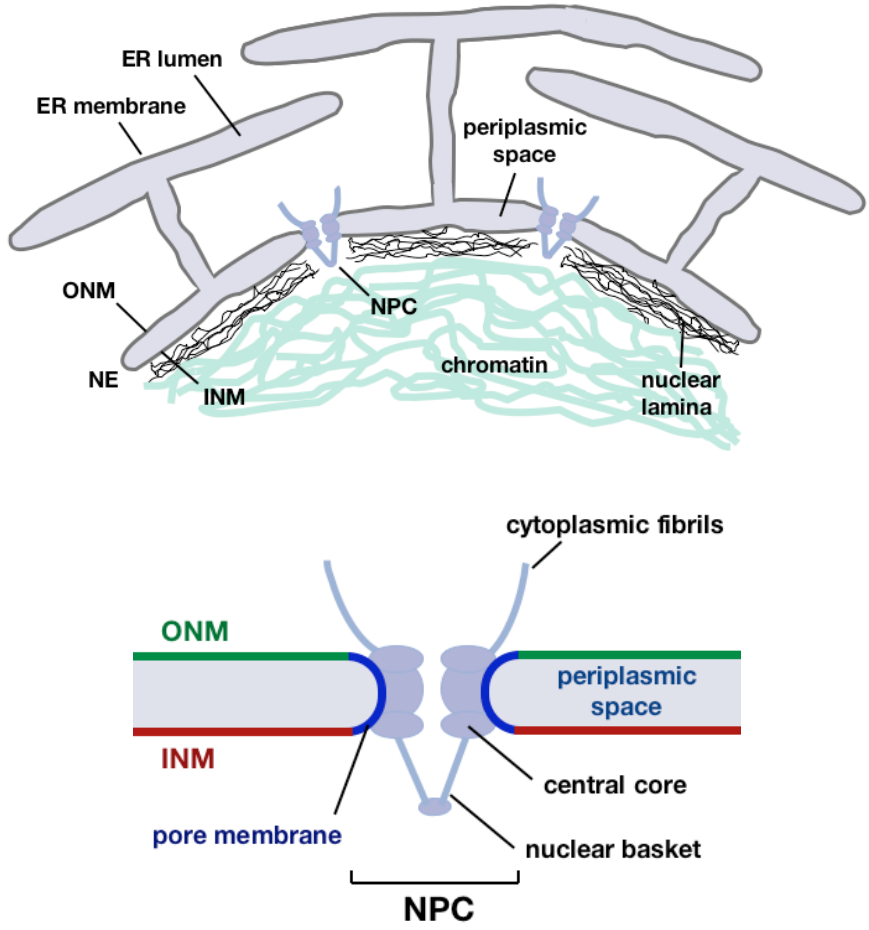

Figure 1. **Schematic diagram of nuclear periphery and associated structures** (*upper panel*). **Nuclear envelope** (*lower panel*). Nuclear envelope (*NE*), inner and outer nuclear membranes (*INM* and *ONM*, respectively), nuclear pore complex (*NPC*), endoplasmic reticulum (*ER*).

Vertebrate cell nuclei undergo "open" mitosis, in which the nuclear lamina and nuclear pore complexes (NPCs) reversibly disassemble, the nuclear membranes merge into the ER, and nuclear proteins are released into the cytoplasm (reviewed in Hetzer et al., 2005). At the end of mitosis, this process in reversed: nuclear membranes re-associate with chromatin, NPCs assemble and nuclear proteins, including lamins, are imported back into the nucleus. An open mitosis in which chromatin is exposed to cytosolic components might be advantageous for metazoan cells by providing additional means for cell-cycle regulation. In contrast, *S. cerevisiae* has a "closed" mitosis in which the NE remains intact. During the closed mitosis, tubulin proteins are imported to allow mitotic spindles to assemble inside the nucleus. Therefore the cell division of *S. cerevisiae* does not include the

molecular unmixing of cytoplasmic and nuclear components and the NE reassembly at the end of mitosis.

1.2 Nuclear lamina

The major component of the nuclear lamina are lamins, type V intermediate filament proteins (reviewed in Stuurman et al., 1998 and Gruenbaum et al., 2005). Lamins have a characteristic structure composed of a long α-helical rod domain flanked by a short N-terminal "head" and a globular C-terminal "tail" domain. The rod domains mediate formation of α-helical coiled-coil dimers, which then assemble into head-to-tail polymers and higher order structures. Lamins are grouped as A-types and B-types. In vertebrates, lamins are encoded by three lamin genes. A-type lamins (lamin A, C, AΔ10 and C2) are encoded from *LMNA* gene and B-type lamins are encoded by LMNB1 (encoding lamin B1) and LMNB2 (encoding lamins B2 and B3). The different forms result from alternative splicing. B-type lamins are expressed in all cells during development and are essential for cell viability, while A-type lamins are expressed in a tissue specific manner. *Drosophila* genome has two lamin-encoding genes, lamin Dm_0 and lamin C, which exhibit some characteristics of vertebrate B and A type lamins, and *C. elegans* possesses only one lamin-encoding gene (Cohen et al., 2001). Little is known about how lamins assemble in the nucleus, for instance, it is unclear whether A and B type lamins co-polymerize, or whether peripheral lamins and lamins localized to the nuclear interior form similar or different structures (Gruenbaum et al., 2005).

Lamins and lamin associated proteins play numerous roles in the cell. In *lmna* null cells, heterochromatin is lost from the nuclear periphery, indicating that lamins are important for attachment of heterochromatin to the NE (Gruenbaum et al., 2005). This attachment could be due to direct binding of lamins to chromatin or indirect association via INM proteins. Also, nuclei of *lmna* null cells have impaired responses to mechanical stress, indicating that lamins are essential for maintaining the mechanical stability of the nucleus (Gruenbaum et al., 2005). Mutations in genes encoding A-type lamins and lamin-binding proteins emerin and LBR cause a wide range of human diseases, collectively called laminopathies, which include premature aging syndromes, myopathies, neuropathies and lipodystrophies (Capell and Collins, 2006).

1.3 Inner nuclear membrane proteins

Inner nuclear membrane (INM) proteins are involved in various processes, including reassembly of nucleus after mitosis, positioning of nucleus within cells, mechanically connecting the nucleoskeleton to cytoskeleton, and gene expression (reviewed in Gruenbaum et al., 2005). Around 15 INM proteins have been characterized in mammalian cells. In addition to these proteins, two genomic approaches identified 67 new putative integral membrane proteins of the nuclear envelope, which were named NETs, for NE transmembrane proteins (Dreger et al., 2001; Schirmer et al., 2003).

A group of metazoan INM proteins share the LEM domain, a 40-amino acids long sequence composed of two helices separated by a short turn. This conserved sequence was named after three mammalian members of this protein family, LAP2, Emerin and MAN1 (Gruenbaum et al., 2005 and Margalit et al., 2007). LEM domain mediates interaction with barrier-to-autointegration factor (BAF), a highly conserved DNA binding protein, which appears to function in heterochromatin formation and NE assembly (Margalit et al., 2007). All LEM proteins tested so far also bind A- and/or B-type lamins, and interactions between lamins, LEM proteins and BAF are proposed to influence chromatin organization during both interphase and mitosis.

In yeast, very few proteins have been characterized as *bona fide* integral INM proteins. There are three integral membrane proteins associated with the NPC: Ndc1, Pom152 and Pom34 (Rout et al., 2000). Prm3 is an INM protein with one putative membrane-spanning segment, required for fusion of nuclear envelopes during mating of yeast cells of opposite mating types (Beilharz et al., 2003; Heiman and Walter, 2000). Two integral INM proteins, Heh1 (also called Src1) and Heh2 share homology with mammalian integral INM proteins of the LEM family, MAN1 and Lem2 (King et al., 2006). Heh1/Src1 is a protein involved in sister chromatid segregation (Rodriguez-Navarro et al., 2002) and the function of Heh2 has not been characterized.

Several integral membrane proteins in yeast exhibiting a typical ER localization have been reported in the literature as NE/ER components, although the experimental evidence for their localization to the INM is missing. Such conclusions are presumably made based on the specific intracellular distribution of ER membranes in yeast; when examined by fluorescence microscopy, yeast ER membrane has a characteristic structure consisting of a nuclear rim staining, which corresponds to the ONM and perinuclear ER, and a peripheral cell staining close to the plasma membrane, which corresponds to the cortical ER. However, proteins exhibiting such localization do not necessarily localize to the INM as well. A possibility that a protein with an intracellular distribution typical of ER membrane also localizes to the INM membrane has to be additionally examined, for instance

by using immuno-electron microscopy. On the contrary, localization of an integral membrane protein exclusively to the nuclear rim is indicative of either ONM or INM localization. The distinction between the two nuclear membranes can be made using immuno-electron microscopy. Integral INM proteins also exhibit typical solubilization characteristics; unlike ER membrane proteins, INM proteins are not extractable by non-ionic detergents, like Triton X-100. To examine whether a NE protein associates with the NPC in yeast, a *nup133Δ* mutant is often used. In this mutant, NPC associated proteins cluster in one or two distinct spots in the NE, while proteins that are not associated with NPC remain evenly distributed in the NE (Doye et al., 1994; Pemberton et al., 1995).

1.4 Nuclear pore complex

At the sites where ONM and INM join, nuclear pore complexes (NPCs) mediate bidirectional transport of molecules across the NE (Figure 1, reviewed in Fried and Kutay, 2003 and Tran and Wente, 2006). Each NPC is built of 30 different proteins, called nucleoporins or Nups. Yeast and metazoan NPCs exhibit a striking degree of structural and functional similarity, and considerable conservation can be found in most individual Nups. A mammalian NPC consists of at least 400 individual proteins that assemble into a 60 MDa complex (Cronshaw et al., 2002). Yeast NPCs are smaller, with an estimated molecular mass of 45 MDa (Rout et al., 2000). At the pore membrane, there are three integral membrane proteins of the NPC: Ndc1, Pom152 and Pom34 in yeast, and Ndc1, gp210 and Pom121 in vertebrates (Tran and Wente, 2006). Around one-third of nucleoporins contains Phe-Gly (FG) repeats that are separated by spacers of various lengths (Cronshaw et al., 2002; Rout et al., 2000). These so called FG-Nups are believed to play an important role in nuclear transport. The architecture of a NPC can be divided into three basic elements: the nuclear basket, the central core and the cytoplasmic filaments (Figure 1). Viewed along the axis of its central channel, the NPC exhibits an octagonal symmetry. Consistently, each Nup is thought to be present in at least eight copies, and many of them are present at higher copy number (Cronshaw et al., 2002; Rout et al., 2000).

NPC is a dynamic structure. It has been demonstrated that residence time of Nups at the NPC varies; some Nups associate very stably with the NPC, and these are proposed to function as its structural components, while other Nups associate only transiently, with residence times limited to minutes or seconds (Rabut et al., 2004). In metazoan cells NPCs reversibly disassemble during the open mitosis, which entails additional mechanisms involved in NPC re-formation (Hetzer et al., 2005). Even some yeasts exhibit a certain degree of mitotic NPC disassembly, potentially analogous to the first

stage of metazoan NE breakdown (Hetzer et al., 2005). It is thought that this partial NPC disassembly in fungi allows nuclear access of several normally cytoplasmic proteins, such as tubulin, in order to allow assembly of mitotic spindles within the nucleus.

1.5 Transport through the nuclear pores

Small molecules like ions and metabolites can freely diffuse through the NPCs, however, proteins and RNA molecules require specific transport receptors and input of energy to be carried across the NPC (reviewed in Tran and Wente, 2006). Transport receptors and other proteins implicated in transport share a striking degree of conservation between yeast and metazoa. The largest class of nuclear transport receptors, which mediates the majority of nuclear transport is the superfamily of importin β related proteins, or karyopherins. Members of this superfamily are also called importins and exportins, reflecting their function in nuclear import or export, respectively. There are 14 members of this family in yeast and more than 20 in metazoa. Transport receptors recognize the cargo molecules by the presence of specific sequences, transport signals. These sequences function as import or export signals, and are also called nuclear localization signals (NLS) or nuclear export signals (NES). Transport signals can be recognized by the importins or exportins directly, or by adaptor proteins, which also have the ability to bind transport receptors. Most transport signals do not fit a well-defined consensus, considerable deviations can occur, and thus the prediction of these signals by bioinformatics tools in not always reliable.

Even though importins and exportins carry the cargo through the NPC presumably by the same mechanism, unidirectional transport of a molecule is possible due to the asymmetric distribution of a small GTPase RanGTP in cytoplasm and nucleus, and the opposite requirements for cargo binding and release in these two environments. RanGTP is scarce in cytoplasm due to efficient GTP hydrolysis facilitated by the presence of Ran-specific GTPase activating protein, RanGAP in this compartment. As RanGAP protein is confined solely to cytoplasm, RanGTP hydrolysis in the nucleus is extremely slow. Additionally, the localization of Ran-GTP-exchange factor (RanGEF) is restricted to the nucleus. Thus, the differential localization of RanGAP in the cytoplasm and RanGEF in the nucleus ensures that the levels of RanGTP are high in the nucleus. This asymmetric distribution of RanGTP forms the basis for directional transport across the NE. Importins, transport receptors that import cargo into the nucleus, bind their cargo in the cytoplasm, the environment free of RanGTP, and release their cargo in the nucleus, the environment abundant with RanGTP. The opposite is true for exportins, the transport receptors that export proteins from the nucleus to the cytoplasm.

The interactions of karyopherins with FG repeats are essential for cargo transport through the NPC (Fried and Kutay, 2003). Several models have been proposed to explain the mechanisms of active transport through the NPC and the role of FG repeats. Recent findings that FG repeats interact with each other, forming hydrogels in vitro support a model which proposes that interactions between adjacent FG repeats form a protein meshwork at the nuclear pore (Frey et al., 2006; Ribbeck and Gorlich, 2002). This meshwork functions as a permeability barrier, allowing the passage of only small molecules. According to this model, transport receptors can overcome the size restriction because their binding to FG repeats out-competes inter-FG-repeat interactions and thus transiently opens adjacent regions of the meshwork.

1.6 Sorting of integral inner nuclear membrane proteins

As the ONM is contiguous with the ER membrane, integral membrane proteins destined for the INM are able to diffuse from the site of their insertion into the ER membrane to the cytoplasmic side of the nuclear pores. To reach the INM, these proteins have to pass through the nuclear pore membrane. One of the intriguing biological questions is how proteins destined for the INM pass the NPC "obstacle" and reach their destination. The "diffusion-retention" model (reviewed in Holmer and Worman, 2001), proposes that proteins destined for the INM move from the ONM to the INM by diffusion in the pore membrane. On their way to the INM, extralumenal domains of the INM proteins are thought to pass through the lateral aqueous channel in the NPC, situated between the NPC and the nuclear pore membrane. According to this model, once INM proteins reach the INM, they are retained there via interaction with the nuclear components, such as lamins and chromatin.

However a recent study demonstrated that transport of integral membrane proteins to the INM requires energy, indicating that in addition to passive diffusion through the nuclear pore membrane, an active transport may exist (Ohba et al., 2004). Consistently, two studies in yeast have identified the presence of NLS motifs in the extralumenally oriented hydrophilic tails of INM proteins Prm3, Heh1 and Heh2 and demonstrated that INM targeting is dependent on their NLS sequences and a functional RanGTPase cycle (Beilharz et al., 2003; King et al., 2006). This active transport seems to be specifically mediated by importin α /importin β karyopherins (King et al., 2006). Intriguingly, these findings suggest that the extralumenal hydrophilic domain of INM proteins might be actively transported through the NPC by mechanisms similar to those used by soluble cargoes. Active karyopherin-mediated transport of INM proteins is also supported by data from a study in a metazoan system showing that a

membrane-associated importin α homologue, which can be found adjacent to the ER translocon, specifically associated with a viral INM-sorting motif as it emerged from the ribosome, and remained associated to it after the release from the ER translocon (Saksena et al., 2006).

To conclude, although it remains possible that diffusion-retention mechanisms could account for the INM localization of certain proteins, especially those with small extralumenal domains that could diffuse through the lateral channel in the NPC, the available data indicate that at least some proteins are targeted to the INM by an active, importin α -dependent nuclear import. Such an import likely involves local NPC rearrangements to accommodate the extralumenal hydrophilic domains of INM proteins. Accordingly, NLS-like sequences appear to be present in many integral INM proteins, both in mammalian cells and in yeast (King et al., 2006).

2. NUCLEAR ENVELOPE AND TRANSCRIPTIONAL REGULATION

Spatial organization of chromatin in the nucleus is non-random, and numerous examples suggest that intranuclear positioning of genes can provide an additional level of transcriptional control (Marshall, 2002). Studies in yeast have provided a useful model for how nuclear organization can affect transcription. Due to enrichment of heterochromatin and silencing factors in the perinuclear area, the nuclear periphery has long been viewed as a solely transcriptionally repressive zone. However, recent data has indicated that the nuclear periphery is not generally repressive, but could be differentiated into at least two zones: a silencing and a non-repressive. Relationship between the NE and transcriptional repression and activation is discussed in the next two sections. Moreover, several INM proteins in metazoan cells have been found to directly influence transcription by regulating the activity of specific transcription factors. In the last section of this chapter, details regarding the regulatory mechanisms involving INM proteins are discussed.

2.1 Nuclear envelope and transcriptional repression - Silencing in yeast

Silenced chromatin in *S. cerevisiae* is analogous to heterochromatin in organisms such as metazoans and plants (reviewed in Rusche et al., 2003). Silencing differs from gene specific repression; in gene specific repression, a particular promoter is specifically targeted for repression, while silencing blocks the expression of genes within the silenced domain irrespective of which transcriptional activator or RNA polymerase is used. There are two main silenced regions in the *S. cerevisiae* genome: the two silent mating type HM loci (HML and HMR) and the telomeres. The rest of the *S. cerevisiae* genome appears to be largely free from silencing (Rusche et al., 2003).

The silent mating type loci are flanked by specific DNA sequences called silencers that are required for transcriptional repression of these genes. Silencing of telomeres and HM loci is dependent on four silent information regulators, or Sir, proteins. Sir proteins are recruited to the silencers and telomeric repeats by specific DNA-binding factors, and following recruitment to these nucleation sites, Sir proteins assemble into a complex. Subsequently, Sir-complexes spread along nucleosomes by binding to underacetylated histones, and a highly ordered and compact structure is formed that restricts transcription. Exact mechanisms by which silenced chromatin inhibits transcription are largely unknown.

In yeast, silenced telomeric domains and HM loci cluster at the nuclear periphery in discrete foci that are far fewer than the number of chromosomal ends (Taddei and Gasser, 2004). In this perinuclear area, Sir proteins are accumulated at a high local concentration. Perinuclear telomere anchoring does not appear to occur via nuclear pores, since NPCs can be clearly distinguished from most telomeric foci. Additionally, in a *nup133Δ* mutant, which exhibits a dramatic clustering of nuclear pores, telomeres remain evenly distributed in a nuclear rim (Taddei and Gasser, 2004). Thus the silenced chromatin localizes close to the NE, but preferentially in the nuclear pore-free areas.

Reporter genes that are inserted adjacent to the telomeres are silenced by a process termed telomere position effect (Gottschling et al., 1990). Conversely, moving a reporter flanked by silencer elements away from the telomere and to the more internal positions on the chromosome, leads to derepression (Maillet et al., 1996). Interestingly, impaired silencing due to the internal nuclear position of the locus could be overcome by either overexpression of Sir proteins, or by mutations that abolish sequestration of Sir proteins at the telomeres. Both of these interventions lead to disperse distribution of Sirs throughout the nucleus. These findings suggested that the repressive transcriptional effect of the telomeres could be due to telomeric sequestration of Sir proteins, and led to the proposal that telomere clustering at the nuclear periphery promotes silencing by maintaining a high local concentration of Sir proteins (Cockell and Gasser, 1999; Maillet et al., 1996).

Sternglanz laboratory experimentally addressed the question whether a locus can become transcriptionally repressed by physical attachment, or anchoring, to the NE (Andrulis et al., 1998). HMR silencer (see above) normally consists of three elements, which are specific DNA sequences that nucleate assembly of Sir complexes. The deletion of any two elements leads to full derepression of the HMR locus (Brand et al., 1985). In the experiments done by (Andrulis et al., 1998), a truncated HMR locus containing only one silencer element, and thus derepressed, was artificially anchored to the NE via a membrane-spanning protein. Strikingly, NE tethering of this locus restored Sir-mediated silencing. This led to the conclusion that NE tethering of an HMR locus with a crippled silencer compensates for the weak nucleation site by placing it in a zone of high Sir protein concentration. In accordance with this idea, NE tethering of a reporter in a mutant that is not able to sequester Sir proteins in perinuclear pools does not confer transcriptional repression (Taddei and Gasser, 2004). However, NE tethering of an HMR locus lacking all three silencer elements was not sufficient to repress transcription, the repression required the presence of at least one silencer element in *cis* (Andrulis et al., 1998). The view that mere NE anchoring is not sufficient for silencing is additionally supported by findings that in some silencing defective mutants, telomeres and HMR locus are transcriptionally derepressed despite

their perinuclear localization (Gartenberg et al., 2004; Taddei et al., 2004; Tham et al., 2001).

In conclusion, gene localization of a locus close to the NE is thought to promote silencing due to high levels of Sir proteins at the nuclear periphery. However, mere anchoring of a locus to the NE is not sufficient for transcriptional repression, the presence of at least a weak silencer element in *cis* is required to nucleate Sir-dependent silencing.

2.2 Nuclear envelope and transcriptional activation

One of the earliest observations that the nuclear periphery might not represent only transcriptionally repressive environment came from a study on mouse L cells, which revealed that active genes localized to the nuclear periphery (Hutchison and Weintraub, 1985). More recent data supporting this view came from studies on chromatin boundary activities. Chromatin boundary activity (also called insulator activity) is defined as the ability of a DNA sequence or an associated protein to protect a gene from the action of a distal enhancer, or from the repressive effects of nearby heterochromatin (reviewed in West et al., 2002). A genetic screen was designed in order to identify proteins with chromatin boundary activities that block propagation of heterochromatin by mechanisms other than transcriptional activation (Ishii et al., 2002). Surprisingly, the results indicated that the propagation of heterochromatin can be blocked by physical tethering of the locus to the nuclear pore basket. This tethering can be executed directly through association of a gene with the nuclear pore basket protein Nup2, or indirectly through proteins involved in nuclear import and export that dock to the inner nuclear pore basket during translocation.

Consistent with this observation, a genome wide ChIP analysis of NPC- and transportin-bound loci identified that many of the nuclear pore associated proteins preferentially bound to highly transcribed genes (Casolari et al., 2004). Interestingly, a set of proteins involved in the nuclear transport was found frequently associated with the genes with binding sites for Rap1 (repressor-activator protein 1), and this association appeared to be independent of their transcription state, as both highly and lowly transcribed genes with Rap1 sites were preferentially bound. Rap1 is a DNA binding protein that functions as both transcriptional activator and repressor (reviewed in Shore, 1994). In addition to its role in transcriptional activation, it also plays a crucial role in the recruitment of Sir proteins to HM loci and telomeres.

Genetic and biochemical approaches indicate that Rap1, together with its two co-activator proteins, associates with the nuclear pores (Menon et al., 2005), findings that are consistent with the ChIP analysis (Casolari et al.,

2004). Based on these results and contrary to the common view that genes are activated by recruitment of transcription activators to the chromatin by relatively free diffusion within the nucleus, Rap1 target genes, and possibly many other genes, appear to be activated by moving to the transcriptional activators assembled at the nuclear pores. To make the distinction from the previous models of transcriptional activation, authors termed this mechanism reverse recruitment (Menon et al., 2005).

However, as mentioned earlier, NPC is a dynamic structure and some of its components are very mobile (section 1.4). For instance, Nup2 is a mobile nucleoporin with a soluble nucleoplasmic pool (Dilworth et al., 2001). Thus, it is not clear whether all reported interactions of proteins and gene loci with the NPC components are restricted to the nuclear periphery. Nevertheless, in accordance with the preferential association of many NPC associated proteins with a subset of transcriptionally active genes (Casolari et al., 2004), it has been found that upon activation, loci of galactose and α-factor inducible genes translocate from the nuclear interior to the periphery. Consistently, ChIP experiments revealed that these genes associated with the NPC components in an inducer-dependent manner (Casolari et al., 2005; Casolari et al., 2004). Thus accumulating data suggests that the gene recruitment to the nuclear envelope may play a role in gene activation and clearly indicate that the nuclear periphery is not a generally repressive zone.

Several studies have tried to address the molecular mechanisms of transcription-induced gene translocation to the NPC, and together the data indicate that different mechanisms may be used for different genes. For instance, NPC association of some genes has been shown to be RNase sensitive, indicating that their perinuclear recruitment is dependent upon mRNA transcripts (Casolari et al., 2005), while translocation of some other loci is mRNA-independent (Casolari et al., 2005; Dieppois et al., 2006). The latter finding suggests that the interaction of genes with the NPC components might be an early event in transcriptional activation that does not require transcripts.

A major unresolved question is whether the association of certain genes with the nuclear periphery represents an obligatory step in their transcriptional activation, or whether it is merely a consequence of it. The available data indicate that, at least for some genes that have been tested, translocation to the nuclear periphery is not an obligatory step in the transcription; in several mutants with impaired nuclear periphery anchoring, the mRNA levels remained unaffected (Cabal et al., 2006; Dieppois et al., 2006). Based on these data, localization to the nuclear periphery appears to be a consequence rather than a cause of transcriptional activation. Therefore it has been proposed that a peripheral gene positioning and their association with the NPC might play a more important role in mRNA processing and nuclear export. However, due to fast diffusion motion of RNA in the nucleus,

it is not sure whether the position effect of a gene relative to the NE has any impact on the export efficiency of its mRNA (Gorski et al., 2006). It has been shown that mRNA molecules of peripherally localized genes exit the nucleus through all available nuclear pores, and not only through the nearest ones (Shav-Tal et al., 2004). Thus, the functional role of gene association with the NPC remains to be clarified.

In conclusion, transcription-induced gene translocation to the nuclear periphery has been observed for a number of genes in *S. cerevisiae* and there are indications that a similar phenomenon exists in metazoan organisms (Brown and Silver, 2007). However, association between NPC and actively transcribed genes should not be considered as a general feature or an integral part of all gene expression in *S. cerevisiae*; the fact that only a subset of active genes has been found at the nuclear periphery indicates that other genes may be regulated in another way. Moreover, the functional significance of gene recruitment to the NPC is not clear yet and needs to be elucidated.

2.3 Regulation of transcription factor activity by nuclear envelope proteins

In last several years, a few metazoan INM proteins have been found to influence the activity of transcription factors. The examples include A-type lamins and three members of the LEM family (see Chapter 1): LAP2 beta, emerin and MAN1. Due to similarities to my work presented in Chapter 4, the examples of MAN1, lamin A/C and LAP2beta and the transcription factors they regulate are described in more detail.

MAN1-mediated regulation of Smads

Smad transcription factors are downstream targets of transforming growth factor β (TGFβ) signaling (reviewed in Massague et al., 2005). Smads undergo constant nucleo-cytoplasmic shuttling, however, in non-induced cells Smads predominantly localize to the cytoplasm, presumably due to the action of cytoplasmic Smad-binding factors. Members of TGFβ superfamily of cytokines bind to membrane receptors and induce direct phosphorylation of five receptor-regulated Smad (R-Smad) factors. Receptor-mediated phosphorylation of R-Smads decreases their affinity for cytoplasmic anchors and increases their affinity for nuclear factors, leading to the nuclear accumulation of these factors. Activated R-Smads form complexes with a common partner for all Smads, Smad 4. In the nucleus, these complexes interact with various co-activators, bind to DNA and regulate transcription of hundreds of target genes that control numerous cellular processes, including proliferation, differentiation and apoptosis. Nuclear R-Smads undergo dephosphorylation that is followed by the dissociation of R-Smad-Smad4

complexes and their subsequent export to the cytoplasm. Thus, R-Smads can be repeatedly phosphorylated and translocated to the nucleus, as long as TGFβ receptors remain active. Activity of Smad proteins is controlled at many different levels, including interaction of TGFβ receptor with R-Smads, formation of R-Smad-Smad4 complexes and post-translational modifications.

Recently, several independent studies on *Xenopus* and mammalian systems have found that the INM protein MAN1 functions as a negative regulator of Smad-mediated signaling (Hellemans et al., 2004; Lin et al., 2005; Osada et al., 2003; Pan et al., 2005; Raju et al., 2003; reviewed in Worman, 2006). MAN1 is a 100 kDa integral protein of the INM with two membrane-spanning segments, and nucleoplasmically oriented N- and C-terminal tails (Lin et al., 2000; reviewed in Gruenbaum et al., 2005). Overexpression of MAN1 inhibits Smad-dependent transcription in a dose dependent manner, and accordingly, downregulation of MAN1 produces an opposite effect (Hellemans et al., 2004; Lin et al., 2005; Osada et al., 2003; Pan et al., 2005; Raju et al., 2003). MAN1 directly interacts with R-Smads at the inner nuclear membrane, independently of the induction by TGFβ and their phosphorylation status (Lin et al., 2005; Osada et al., 2003; Pan et al., 2005; Raju et al., 2003). Although R-Smads are predominantly localized to the cytoplasm in the absence of TGFβ signaling, a perinuclear staining that co-localizes with MAN1 is present, suggesting that a pool of R-Smads may associate with the nuclear membrane. Even after the TGFβ-induced translocation of R-Smads into the nucleus, there is still a detergent non-extractable fraction at the nuclear membrane that co-localizes with MAN1 (Pan et al., 2005).

MAN1 mediated inhibition of Smad activity could be explained by a model in which MAN1 sequesters R-Smads at the INM. The sequestration of R-Smads at the INM may reduce availability of R-Smads that can be activated in the cytoplasm, or it can prevent formation of transcriptionally active complexes in the nucleus. Consistently with the latter possibility, MAN1-associated R-Smads are not found associated with Smad4 (Pan et al., 2005).

Lamin A/C-mediated regulation of AP-1

AP-1 is a dimeric transcription factor, composed of mainly members from the Fos and Jun protein families (reviewed in Hess et al., 2004). Fos and Jun heterodimerization is required for high affinity of AP-1 DNA binding. AP-1 participates in the regulation of cellular processes such as cell proliferation, cell differentiation and apoptosis and is activated in response to various signals, including cytokines and growth factors. AP-1 activity is regulated at several different levels, including transcription of genes encoding its subunits, post-translational modifications, protein stability, dimer composition and interactions with various regulatory proteins.

Recently, it has been found that the activity of AP-1 can be negatively regulated via direct interaction of c-Fos with A-type lamins A and C, two spliced variants of LMNA gene (see chapter 1.2, Ivorra et al., 2006). In quiescent serum deprived cells, AP-1 subunit c-Fos localizes mainly to the NE, in a strictly lamin A/C-dependent manner. NE localization of c-Fos correlates with lack of AP-1 binding to the target DNA sequences. After addition of serum to the cells, expression of c-Fos is transcriptionally upregulated and a massive nuclear accumulation of c-Fos can be observed, which correlates with AP-1 binding to DNA. Few hours after serum stimulation, nucleoplasmic c-Fos is downregulated and residual nuclear c-Fos can be observed at the NE. In these cells, AP-1 DNA binding is lost. Thus lamin A/C-dependent NE localization of c-Fos directly correlates with the lack of AP-1 DNA binding, indicating that lamin A/C may facilitate the suppression of AP-1 in serum deprived cells by sequestering residual c-Fos at the nuclear periphery. Consistently, it has been shown that overexpression of lamin A/C results in decreased AP-1 DNA binding due to hindered interaction between c-Fos and c-Jun.

LAP2β-mediated regulation of E2F

Integral INM protein LAP2β (Gruenbaum et al., 2005) has also been implicated in negative regulation of transcription factor activity. When overexpressed, LAP2β negatively affects activity of heterodimeric E2F-DP3 transcription factor (Nili et al., 2001; Somech et al., 2005). Similarly to MAN1 and lamin A/C, the mechanisms of this regulation appear to include sequestration of the transcription factor at the INM, which may inhibit transcription due to a spatial separation from the target promoters. Unlike MAN1 and lamin A/C, LAP2β does not directly interact with E2F-DP3 transcription factor, rather the interaction is indirect via mGCL (mouse germ-cell-less), a conserved protein with transcription-repressing ability (de la Luna et al., 1999; Nili et al., 2001). In addition, LAP2β mediated downregulation of E2F-DP3 function may also involve modulation of high order chromatin structure. This idea is based on the finding that histone deacetylase HDAC3 interacts with LAP2β, and when overexpressed, LAP2β is able to direct HDAC3 to the NE in a dose dependent manner (Somech et al., 2005). In this model, while still bound to the chromatin through its target promoters, E2F-DP3 factor is recruited to the NE via indirect interaction with LAP2β. Once at the nuclear periphery, E2F-DP3-bound chromatin is modulated to the repressive form by the action of HDAC3, which is also recruited to the NE via interaction with LAP2β.

Common to these three cases of INM proteins regulating transcription factor activity, MAN1, lamin A/C and LAP2β, is sequestration of a transcription factor at the NE, either via direct interaction, or indirectly through another

protein. The sequestration could inhibit activity of a transcription factor by one or a combination of the following reasons: physical separation of a factor from the target promoters, hindered formation of transcriptionally active complexes with other factors, or, as may be true for LAP2β, co-recruitment of target promoters and chromatin remodeling enzymes to the nuclear periphery.

3. AMINO ACID INDUCED SIGNALING - SPS SENSOR PATHWAY

3.1 SPS-sensor signaling pathway - overview

The regulation of amino acid uptake in yeast offers an attractive system to study mechanisms of signal transduction. Yeast cells need amino acids for protein biosynthesis and must either import or synthesize the 20 amino acids found in proteins. Even though yeast can synthesize all amino acids, when available, yeast cells prefer to import them and possess sophisticated and complex system of transporters. Amino acids are taken up by the amino acid permease (AAP) protein family, which includes 18 core members. AAPs share sequence homology and a common structure of twelve membrane-spanning segments, and cytoplasmically oriented N- and C- termini (Gilstring and Ljungdahl, 2000). Each amino acid permease has its own substrate specificity profile with distinct affinities for different amino acids (Regenberg et al., 1999). Notably, import of amino acids into cells has to be regulated, as imbalanced or high internal amino acid concentrations negatively affect metabolic regulation and impair cell growth. Functional expression of amino acid permeases is regulated at the transcriptional and post-transcriptional level, including processes such as intracellular protein sorting and degradation (Forsberg et al., 2001).

A signaling pathway from the plasma membrane to the nucleus is involved in sensing amino acid availability in the environment and regulating their uptake by inducing the transcription of eight AAPs (Forsberg and Ljungdahl, 2001). Extracellular amino acids are recognized by a receptor in the plasma membrane, the integral membrane protein Ssy1 (Wu et al., 2006). Ssy1 is also a member of the AAP family, however it is distinct from all other members by having an unusually long cytoplasmic N-terminal tail and two extended hydrophilic loops facing the extracellular environment (Didion et al., 1998). Despite homology with the members of AAP family, Ssy1 does not transport amino acids (Didion et al., 1998; Iraqui et al., 1999; Wu et al., 2006), but together with two peripherally associated membrane proteins, Ptr3 and Ssy5, forms the Ssy1-Ptr3-Ssy5 (SPS) sensor of extracellular amino acids (Klasson et al., 1999).

The only known downstream effectors of the SPS-sensor are Stp1 and Stp2, two homologous Zn^{2+} finger transcription factors, which exhibit overlapping and partially redundant functions (Andréasson and Ljungdahl, 2002; de Boer et al., 2000; Nielsen et al., 2001; Wang and Hopper, 1988; Wang et al., 1992). Stp1 and Stp2 are latent cytoplasmic transcription factors synthesized with 10 kDa N-terminal regulatory domains crucial for the regulation of their activity. In the absence of extracellular amino acids, the N-terminal domains prevent nuclear accumulation of Stp1 and Stp2

(Andréasson and Ljungdahl, 2002). Under this condition, Stp1 and Stp2 are diffusely localized throughout the cell, and importantly, do not accumulate in the nucleus (Andréasson and Ljungdahl, 2002). In response to amino acids, latent Stp1 and Stp2 are activated by endoproteolytic removal of their N-terminal domains by the action of the Ssy5, a receptor-activated chymotrypsin like serine protease (Andréasson et al., 2006; Andréasson and Ljungdahl, 2002). The shorter forms of Stp1 and Stp2 efficiently target to the nucleus where they directly bind to specific upstream activating sequences (UAS_{aa}) present within SPS-sensor regulated promoters (Abdel-Sater et al., 2004; de Boer et al., 2000; Nielsen et al., 2001). Gene targets of Stp1 and Stp2 include amino acid permease genes *AGP1*, *BAP2*, *BAP3*, *DIP5*, *GNP1*, *MUP1*, *TAT1* and *TAT2* and the peptide transporter gene *PTR2*. However, the possibility that additional target genes of the SPS sensor pathway may exist cannot be excluded. The mechanism that prevents nuclear accumulation of unprocessed Stp1 and Stp2 is not clear, but it could involve regulation of their nucleo-cytoplasmic transport, or physical anchoring to a cytosolic component. SPS-sensor signaling pathway is schematically depicted in Figure 2.

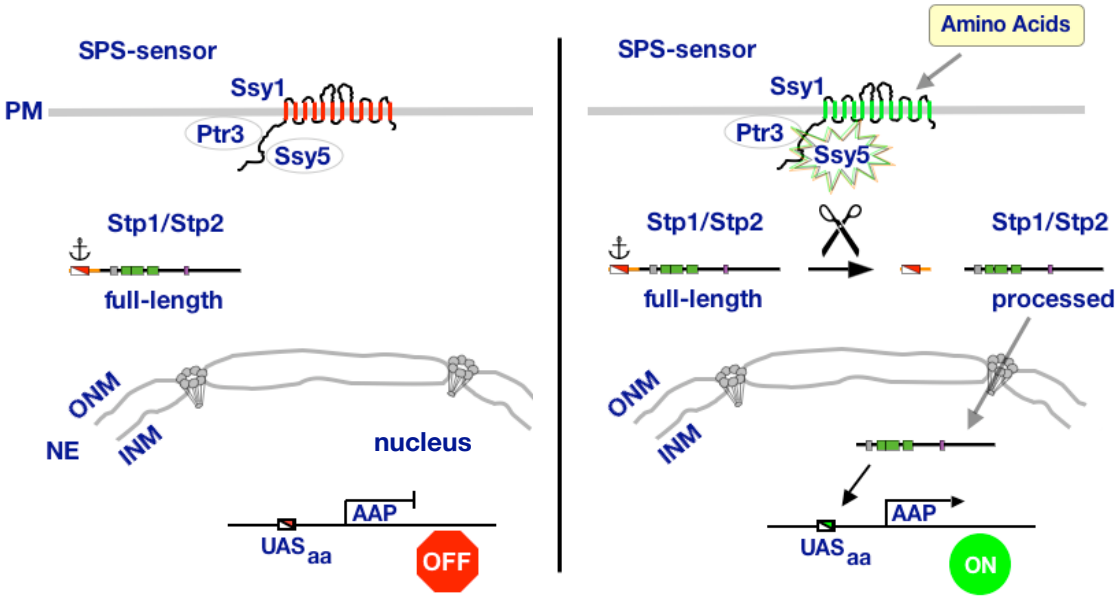

Figure 2. **SPS-sensor signaling pathway**. In the absence of amino acids (*left panel*), Stp1 and Stp2 exist in their full-length forms. The N-terminal regulatory domains of Stp1 and Stp2 (*red and white box*) function as cytoplasmic retention motifs and prevent their nuclear accumulation (*anchor*). In the presence of amino acids (*right panel*), plasma membrane (*PM*) localized SPS sensor is activated (*green*). Ssy5 mediates endoproteolytic processing of Stp1 and Stp2 (*scissors*). The processed forms of Stp1 and Stp2 efficiently target to the nucleus (*arrow*), where they bind to the UAS_{aa} elements within AAP gene promoters and activate transcription of AAP genes (*ON*).

3.2 N-terminal regulatory domains of Stp1 and Stp2

Stp1 (species specific tRNA processing) was originally identified as a high-copy facilitator of tRNA processing which on amino acid-containing medium localizes to the nucleus (Wang and Hopper, 1988; Wang et al., 1992). Stp1 was subsequently shown to be required for amino acid uptake (Jørgensen et al., 1998; Jørgensen et al., 1997). *STP2* was found as a multi-copy suppressor gene that enabled SPS-sensor dependent uptake of branched chain amino acids (de Boer et al., 2000). It was given its name based on homology with *STP1*.

The N-terminal domains of Stp1 and Stp2 contain all the information needed to confer latent behavior in the absence of amino acids and amino acid dependent endoproteolytic activation by the SPS sensor. This has been shown in an experiment in which the N-terminal domain of Stp1 encompassing the first 125 amino acids was fused to an artificial transcription factor composed of the bacterial DNA binding protein LexA and the viral activation domain VP16 (Andréasson and Ljungdahl, 2004). In the absence of any additional sequences, LexA-VP16 efficiently targets to the nucleus, and activates transcription of a reporter gene fused to LexA operators. Remarkably, the fusion protein Stp1$_{(aa\ 1\text{-}125)}$-LexA-VP16 is endoproteolytically processed in an amino acid and SPS sensor dependent manner, which is followed by the reporter gene activation. However, when cells are grown in the absence of amino acids, Stp1$_{(aa\ 1\text{-}125)}$-LexA-VP16 construct exists in its full-length form. The N-terminal domain of Stp1 confers effective nuclear exclusion of this fusion protein and, consequently, the reporter gene fused to LexA operators is repressed.

By sequence comparisons between Stp1 and Stp2 homologues in other fungal species, two conserved regions within the N-terminal domains of Stp1 and Stp2 were identified (Andréasson and Ljungdahl, 2004). Region I is in the middle of the N-terminal regulatory domain and contains many branched chain amino acids. Distinct mutations in this region (*STP1-133*) give rise to a constitutively active factor, suggesting that this region is important for the latent behavior of the full-length factors. A stretch of conserved amino acids more downstream of Region I is essential for the endoproteolytic processing of Stp1. Proteins carrying mutations in this region (*stp1-102*), called Region II, are not cleaved in response to amino acids.

3.3 Dal81 - a nuclear factor shared by three pathways

Dal81 is a nuclear factor required for full induction of SPS sensor regulated AAP genes (Bernard and André, 2001; Iraqui et al., 1999). Dal81 is not specific for the SPS sensor pathway, it is also involved in two other nitrogen source utilization pathways: a pathway for utilization of urea and allantoin (Jacobs et al., 1985; Turoscy and Cooper, 1982) and γ-aminobutyric acid (GABA) (Vissers et al., 1989). In all three pathways, Dal81 functions together with an inducer specific transcription factor, namely Stp1 or Stp2 (see section 3.1), Dal82 (Jacobs et al., 1985) or Uga3 (Andre, 1990) to activate target genes via inducer specific sequences (Abdel-Sater et al., 2004; Talibi et al., 1995; van Vuuren et al., 1991). While the inducer specific factors Stp1, Stp2, Dal82 and Uga3 directly bind to the specific upstream activating sequences (de Boer et al., 2000; Dorrington and Cooper, 1993; Idicula et al., 2002; Nielsen et al., 2001; Noel and Turcotte, 1998), direct binding of Dal81 to these elements has not been demonstrated. Additionally, the pleiotrophic function of Dal81 at different promoters, and the finding that the deletion of the putative DNA binding domain of Dal81 has no effect on the induction of allantoin/urea and GABA catabolizing pathways (Bricmont et al., 1991), suggested that Dal81 exerts its effects independently of direct DNA binding. In a genome-wide chromatin immuno-precipitation study, Dal81 was found associated with many SPS sensor regulated promoters together with Stp1 and/or Stp2 (Lee et al., 2002). This association is most likely indirect, perhaps via Stp1 and Stp2. Consistently, activation via a 21bp UAS_{aa} element from *AGP1* promoter is both Stp1 and Dal81 dependent, indicating that these proteins exert their function via the same sequence (Abdel-Sater et al., 2004).

3.4 Discovery of *ASI1*, *ASI2* and *ASI3*

In order to identify downstream components of the SPS sensor, a genetic screen was designed to isolate mutations that would restore amino acid uptake in SPS sensor deficient cells (Forsberg et al., 2001). Mutants with an impaired SPS sensor do not express amino acid permeases that import leucine with high affinity, thus in amino acid rich medium, leucine uptake is out-competed by the presence of other abundant amino acids. Consequently, *leu2* auxotrophic strains carrying mutations that abolish SPS sensor function, such as *ssy1Δ* or *ptr3Δ,* are unable to grow on amino acid rich medium. The inability to grow is due to their inability to either synthesize or import leucine in amounts sufficient to support growth (Klasson et al., 1999). The synthetic non-growth phenotype of *ssy1Δ leu2* and *ptr3Δ leu2* strains on an amino acid rich medium formed the basis for the *ASI* (amino acid sensor independent)

genetic screen. Spontaneous suppressing mutations in *ASI* genes that restore growth of the starting strains on the amino acid rich medium were isolated.

The *asi* mutations defined two phenotypic classes. Mutations belonging to class I constitutively activated transcription of SPS-sensor regulated genes, while mutations belonging to class II stabilized residual amino acid permeases at the plasma membrane by impairing their targeting to the vacuole. The genes defined by the class I mutations include *ASI1*, *ASI2*, *ASI3*, *TUP1*, *SSN6* and *ASI13*. *TUP1* and *SSN6* encode previously characterized transcriptional repressors that act on many genes (Malave and Dent, 2006). However, *tup1* and *ssn6* mutations did not constitutively activate all SPS sensor regulated promoters, suggesting that Tup1 and Ssn6 repressors are not directly regulated by the SPS sensor pathway. In contrast, recessive mutations in previously uncharacterized *ASI1*, *ASI2* and *ASI3* genes constitutively activated all SPS sensor regulated promoters tested. The recessive nature of *asi1*, *asi2* and *asi3* mutations indicated that the wild type gene products normally function as negative regulators of the SPS sensor pathway. Structural and functional characterization of Asi1, Asi2 and Asi3 is described in detail in the next chapter. Additionally, in the *ASI* screen, a dominant mutation that gave rise to a strong constitutive transcription of AAP genes, *ASI13-1*, was isolated. Cloning of *ASI13-1* revealed that the mutation resided in *STP1*. The *ASI13-1* mutation turned out to be a large in-frame deletion of 58 codons within the corresponding N-terminal domain of Stp1, and this finding formed the basis for the subsequent discovery of latent behavior of Stp1 and Stp2 and their endoproteolytic activation (Andréasson and Ljungdahl, 2002).

4. SUMMARY AND DISCUSSION OF THE RESULTS

4.1 The aim of this thesis

In the *ASI* screen, three novel proteins, Asi1, Asi2 and Asi3, were found as negative regulators of the transcription of SPS sensor dependent genes (Forsberg et al., 2001). Sequence analysis suggested that these proteins are integral membrane components with multiple membrane-spanning segments. *ASI1* and *ASI3* encode homologous proteins sharing 31 % identity and 42 % similarity. Their C-terminal hydrophilic tails end with conserved Zn^{2+}-binding RING domains (Freemont, 1993). Database searches have not identified the presence of any recognizable motif within Asi2.

The aim of this thesis was to find how Asi1, Asi2 and Asi3 exert their negative regulatory function in the SPS sensor pathway.

4.2 Inner nuclear membrane Asi proteins maintain latency of unprocessed Stp1 and Stp2 (Papers I and II)

We anticipated that determining the intracellular localization of Asi1, Asi2 and Asi3 would greatly contribute to the understanding of their function. In subcellular fractionation experiments, Asi1, Asi2 and Asi3 were found to co-fractionate with ER membrane proteins (Paper II). However, our microscopic analysis revealed that all three proteins localize exclusively to the nuclear rim, and by using immuno-electron microscopy we found that they are components of the INM (Papers I and II). The experimentally determined membrane topology of Asi proteins is illustrated in the Figure 3. At the time these results were obtained in our lab, there were no other literature reports of the *bona fide* integral INM proteins in yeast, which made this finding intriguing, especially considering the role of Asi proteins in controlling gene expression.

We initially considered the possibility that Asi proteins act as negative regulators of the SPS sensor pathway by directly repressing AAP promoters in the absence of amino acids. However, this turned out not to be the case; when the N-terminal regulatory domain of Stp1 is fused to an artificial transcription factor LexA-VP16, it is sufficient to confer nuclear exclusion and prevent expression of a gene reporter fused to LexA operators (see section 3.2). Importantly, this repression is strictly dependent upon the presence of functional Asi proteins (Andréasson and Ljungdahl, 2004). Since in this experiment Asi-mediated control is exerted on a completely unrelated DNA sequence (i.e., LexA operators), this result clearly indicated that the function of Asi proteins is not specific for the SPS sensor regulated promoters only.

Moreover, this experiment also demonstrated that Asi proteins function via the N-terminal domains of Stp1 and Stp2.

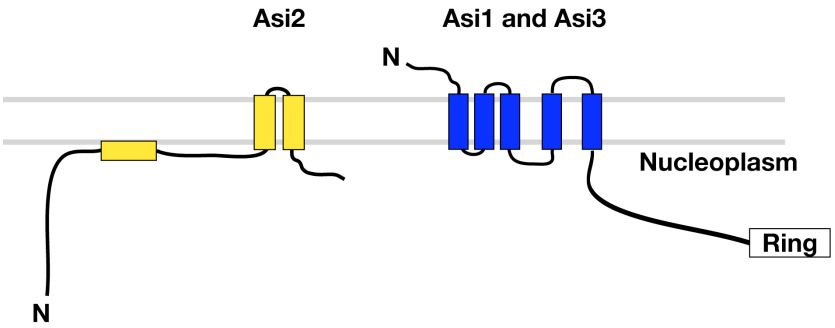

Figure 3. **Experimentally determined membrane topology of Asi, Asi2 and Asi3**.

The finding that Stp1 and Stp2 accumulate in the nucleus in response to amino acids (Andréasson and Ljungdahl, 2002) indicated that activation of AAP genes is controlled by regulating the access of Stp1 and Stp2 to the AAP promoters. We have directly tested this possibility by chromatin immuno-precipitation (ChIP) experiments and found that in wild type cells Stp1 associates with the promoters only after amino acid induced processing (Paper I). Remarkably, we found that constitutive transcription of AAP genes in *asi* mutants is due to the ability of the full-length Stp1 and Stp2 to associate with the promoters (Paper I). Thus, in order to maintain the repressed state of signaling in the absence of extracellular amino acids, the function of Asi proteins is required to restrict promoter access of latent forms of Stp1 and Stp2.

As discussed in Chapter 2, nuclear periphery has often been viewed as a transcriptionally repressive environment. The possibility that in the absence of amino acids AAP genes are silenced by the Sir-dependent mechanisms could be essentially ruled out based on findings that a mere NE tethering of a locus does not result in transcriptional repression unless there is at least a weak silencer element present in *cis* to nucleate silencing (see section 2.1). However, a report that a mammalian integral INM protein LAP2β negatively affects transcription by a mechanism that could include co-recruitment of specific promoters and a chromatin-remodeling enzyme to the nuclear periphery, prompted us to consider the possibility that Asi proteins may function in a similar manner (Somech et al., 2005, see section 2.3). In this model Asi-dependent repression is achieved by the recruitment of the promoters to the nuclear periphery through the interaction of Asi proteins with Stp1 and Stp2 N-terminal domains. Our results from ChIP experiments

and the repression assay clearly exclude this mechanism; opposite of what we would expect if this model was correct, full-length Stp1 and Stp2 or LexA-Stp1$_{(aa\ 2\text{-}69)}$ and LexA-Stp2$_{(aa\ 2\text{-}77)}$ constructs are restrained from binding promoters in an Asi-dependent manner (Paper I).

Crucial to our understanding of the role of Asi proteins was the finding that *asi* mutations do not lead to nuclear accumulation of full-length Stp1 and Stp2. Apparently only a small pool of unprocessed factors is able to enter the nucleus, demonstrating that *asi* mutations do not interfere with the major cytoplasmic retention system (Paper I). Notably, despite low levels of unprocessed Stp1 and Stp2 in the nuclei of non-induced *asi* mutants, ChIP experiments reveal that the levels of Stp1 associated with the promoters in non-induced *asi1Δ* cells are indistinguishable to those observed in induced wild-type cells, in which Stp1 and Stp2 are accumulated in the nucleus (Paper I). Several conclusions can be drawn based on these results. First, as mentioned above, the data indicate that activation of AAP genes is entirely regulated by controlling the access of Stp1 and Stp2 to the promoters. Second, the unprocessed forms of Stp1 and Stp2 function equally well as transcriptional activators if they are able to access the promoters. Third, Stp1 and Stp2 appear to bind to the target promoters with a high affinity, which is consistent with the full activation of AAP promoters in non-induced *asi* mutants. These finding collectively indicate that the system for activation of AAP genes is very sensitive.

In conclusion, the data from Paper I and Paper II reveal that in addition to cytoplasmic retention mechanisms that prevent nuclear accumulation of unprocessed Stp1 and Stp2, an additional negative regulatory system in the nucleus is necessary to ensure the repressed state of signaling in the absence of amino acids. This nuclear system is comprised of three integral INM Asi proteins, which maintain the latent behavior of full-length Stp1 and Stp2 by preventing their access to the target promoters.

An interesting biological question is how Asi proteins are sorted to the INM. Data from several recent reports indicate that targeting of integral membrane proteins to the INM is an active process mediated by the karyopherin transport machinery common to the nuclear import of soluble cargo (Beilharz et al., 2003; King et al., 2006; Saksena et al., 2006, for details see section 1.6). Accordingly, NLS-like sequences can be found in extralumenal regions of many integral INM proteins (King et al., 2006). By sequence analysis, we identified putative NLS signals in the C-terminal tail of Asi3, indicating that, similarly to INM proteins Prm3, Heh1 and Heh2 (see sections 1.3 and 1.6), Asi3 might be targeted to the INM by the same karyopherin-mediated transport mechanism. The inability to identify similar NLS motifs in Asi1 and Asi2 could be merely a consequence of limitations of bioinformatics tools, or could indicate that an independent mechanism for

INM protein targeting exists. Hopefully, future experiments will address mechanisms of Asi protein targeting to the INM.

4.3 Dal81 augments Stp1 and Stp2 mediated signaling (Paper III)

In Paper III we report that the sensitivity of SPS sensor signaling is dependent on Dal81, a nuclear factor involved in induction of three pathways for nitrogen source utilization (see chapter 3.3). In the absence of functional Dal81, AAP promoters remain partially active in Stp1 and Stp2 dependent manner, indicating that Dal81 is not a core component of the SPS-sensor signaling pathway. Similarly, in response to GABA, the inducer specific transcription factor Uga3 is able to induce low levels of *UGA1* gene expression even in the absence of Dal81 (Talibi et al., 1995).

We find that Dal81 becomes stringently required for the expression of AAP genes when the concentration of Stp1 and Stp2 in the nucleus is low, a condition that we can experimentally achieve in non-induced *asi* mutant cells. Thus in the absence of Dal81, *asi* mutations do not lead to constitutive AAP gene activation. This finding suggested that perhaps in *dal81* mutants, Stp1 and Stp2 are not able to efficiently associate with the AAP promoters. We directly tested this possibility by ChIP experiments and, consistent with our phenotypic analysis, we found that loss of functional Dal81 severely impairs binding of Stp1 to the AAP promoters. As mentioned in section 3.3, Dal81 functions via the same UAS_{aa} sequences as Stp1 and Stp2 (Abdel-Sater et al., 2004), however in contrast to Stp1 and Stp2, Dal81 does not bind DNA directly (Bricmont et al., 1991). In a simple model, association of Dal81 with Stp1 and Stp2 enhances their affinity for binding to the UAS_{aa} elements.

Together the data from Paper III demonstrate that Dal81 augments Stp1- and Stp2-mediated transcriptional activation, presumably by increasing the efficiency of Stp1 and Stp2 association with the target promoters. Our findings illuminate the role of the poorly described nuclear factor Dal81 in SPS sensor signaling and suggest that Dal81 might function in a similar way within pathways for utilization of urea, allantoin and GABA. Moreover, our findings add to understanding of the significance of the nuclear Asi-system; in the presence of Dal81 even very low amounts of Stp1 and Stp2 are sufficient to fully activate SPS sensor regulated promoters. Hence, the negative regulatory Asi-system restricting the activity of low amounts of nuclear unprocessed Stp1 and Stp2 is required to prevent inappropriate gene activation.

4.4 A model for Asi mediated control of Stp1 and Stp2 latency

Based on the available data, the entire regulation of the SPS sensor pathway converges on controlling the activity of Stp1 and Stp2. This regulation includes maintenance of their latent behavior in the absence of amino acids, and efficient mobilization upon addition of amino acids to the medium.

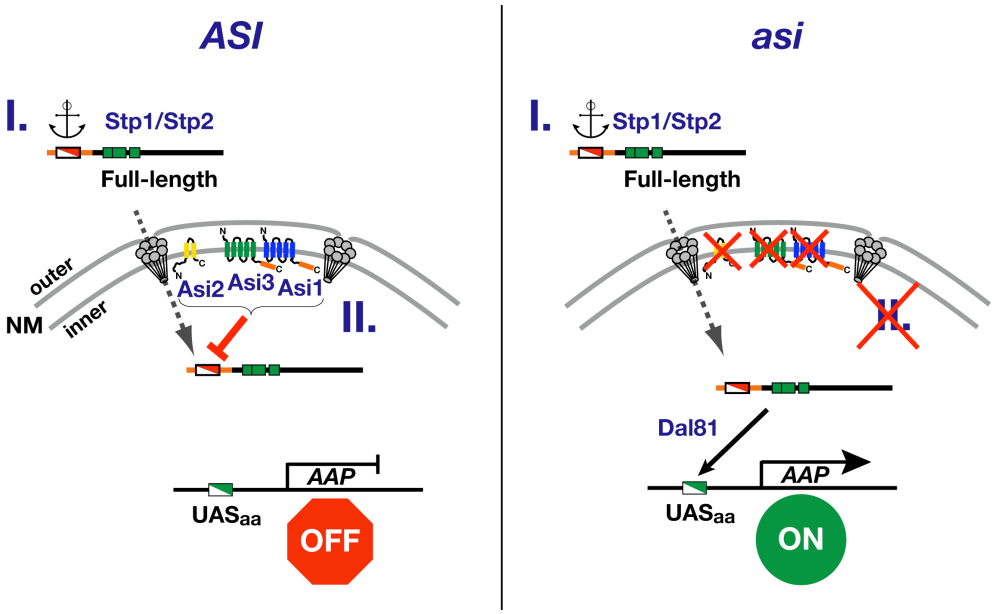

Figure 4. **Model for two-step control of Stp1 and Stp2 latency in the absence of amino acids**. *Left panel*: Two levels of control (*I* and *II*) converge on the N-terminal regulatory domains of Stp1 and Stp2 (*red and white box*) to maintain their latent behavior in the absence of amino acids. The first regulatory mechanism (*I*) retains unprocessed forms of Stp1 and Stp2 in the cytoplasm and prevents their nuclear accumulation (*anchor*). However, low levels of full-length Stp1 and Stp2 escape cytoplasmic retention and enter the nucleus (*dashed arrow*). The second regulatory mechanism in the nucleus (*II.*) restricts promoter access of nuclear unprocessed Stp1 and Stp2. This mechanism is dependent upon complex of three Asi proteins at the inner nuclear membrane (*inner NM*), which sequester full-length Stp1 and Stp2 and impair their association with the AAP gene promoters. *Right panel*: In cells lacking one or all Asi proteins (*asi*), the second negative regulatory mechanism is impaired. Low amounts of full-length Stp1 and Stp2 that enter the nucleus are able to bind to the UAS_{aa} elements and constitutively activate expression of AAP genes. Efficient promoter binding of Stp1 and Stp2 requires the presence of Dal81, hence AAP expression in non-induced *asi* mutants is strictly Dal81 dependent.

Our data is consistent with a model in which the latent behavior of Stp1 and Stp2 is achieved by two levels of control, which both function via Stp1 and Stp2 N-terminal regulatory domains (Figure 4). The first level of control retains the full-length forms of Stp1 and Stp2 in the cytoplasm and prevents their nuclear accumulation. This regulatory step is Asi-independent. Mutations in the conserved Region I of Stp1 (*STP1-133*) abolish this function and result in a constitutively active factor that accumulates in the nucleus

(Andréasson and Ljungdahl, 2004 and Paper III). However, the cytoplasmic retention mechanism appears not to be fully effective in restricting promoter access of unprocessed Stp1 and Stp2. Due to the high affinity of Stp1 and Stp2 for AAP promoters, a characteristic that is dependent on Dal81, even low levels of unprocessed nuclear Stp1 and Stp2 give rise to transcriptional activation. Therefore the second negative regulatory step, which is Asi-dependent, is required to prevent promoter access of unprocessed Stp1 and Stp2 that inappropriately enter the nucleus.

Perhaps the most straightforward mechanism by which Asi proteins could restrict unprocessed Stp1 and Stp2 from accessing promoters is by directly binding and sequestering the full-length forms at the INM. Sequestration at the INM could restrict promoter binding due to impaired association of Stp1 and Stp2 with Dal81 or/and spatial separation from the promoters. There are several examples of metazoan INM proteins that regulate transcription factor activity by mechanisms that appear to include sequestration (Ivorra et al., 2006; Nili et al., 2001; Worman, 2006; for details see chapter 2.3). INM proteins MAN1 and lamin A/C directly bind specific transcription factors at the INM, and this is believed to prevent the formation of transcriptionally active complexes with other components and association of transcription factors with the promoters (Ivorra et al., 2006; Worman, 2006). Additionally, as previously mentioned, negative regulatory role of LAP2β on transcription might also be due to recruitment of a chromatin-remodeling enzyme to the nuclear periphery (Somech et al., 2005). In all these cases, the overexpression of the INM protein results in decreased transcriptional response to the inducer, and accordingly, the down-regulation of the INM protein has an opposite effect. Most similarities with Asi-mediated control could be found in MAN1-dependent regulation of Smad activity (Worman, 2006). However, it is important to note that, in contrast to the effect of *asi* mutations, the downregulation of MAN1 does not lead to constitutive signaling in the absence of the inducer, it increases only induced levels of transcription (Pan et al., 2005). This finding indicates that, in contrast to the function of Asi proteins, MAN1 appears to control the induced, and not basal levels of gene expression.

We tried to experimentally address the possibility that Asi proteins might interact directly with unprocessed Stp1 and Stp2. Using a yeast-two-hybrid approach we tested the interaction between the N-terminal domain of Stp1 and the hydrophilic nucleoplasmic tails of Asi1, Asi2 and Asi3. In each instance, we failed to detect an interaction. The lack of success could reflect the possibility that other factors are involved, or that all three Asi proteins are required for interaction with N-terminal domain of Stp1. The latter possibility would be consistent with a model in which Asi proteins function together in a complex within the INM. The idea of Asi-complex is based on several observations (Paper II). First, mutations in *ASI1, ASI2* and *ASI3* belong to the

same epistasis group; null mutations in any of these genes, as well as all combinations of double and triple mutations give rise to identical phenotypes, indicating that the Asi proteins participate in the same regulatory step. Secondly, all three Asi proteins function to restrict the activity of unprocessed Stp1 and Stp2 under non-inducing conditions. Third, the Asi proteins are integral membrane proteins that co-localize to the inner nuclear membrane. Fourth, Asi1 and Asi3 are homologous, but not redundant proteins. Finally, Asi1 and Asi3 co-purify.

4.5 Future perspectives

Hopefully, future work will increase our understanding of mechanisms controlling the latent behavior of unprocessed Stp1 and Stp2. A crucial issue to resolve is the mechanism of Stp1 and Stp2 nuclear exclusion under non-inducing conditions. Are full-length forms of Stp1 and Stp2 anchored to a yet unidentified cytoplasmic component? Or, is their predominantly cytoplasmic localization a consequence of efficient nuclear export that is dependent on their N-terminal regulatory domains? Moreover, are there other nuclear components required for the latent behavior of Stp1 and Stp2, perhaps working in concert with Asi proteins?

In addition to searches for interaction partners of Asi proteins and the N-terminal domains of Stp1 and Stp2, some of these questions might be addressed using genetic approaches designed to identify other potential negative regulatory components of the SPS sensor pathway. For instance, such approaches could identify a cytoplasmic determinant responsible for Stp1 and Stp2 anchoring, or an additional nuclear protein that restricts function of full-length forms of Stp1 and Stp2.

Since the *ASI* genetic screen was not saturated (Forsberg et al., 2001; see chapter 3.4), a genetic approach based on the same experimental design, but using duplicated *ASI1*, *ASI2* and *ASI3* genes in order to avoid isolation of mutations in these genes could be fruitful. This genetic screen would most likely identify loss-of-function mutations in genes encoding negative regulatory components of the SPS sensor pathway. However, this screen might overlook genes whose products are essential for cell viability or exhibit redundant function in SPS sensor signaling. Such components could be identified in a multi-copy suppressor screen of *asi* mutations looking for components whose overexpression abolishes constitutive AAP gene activation in *asi* mutants.

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