In yeast the homologous transcription factors Stp1 and Stp2 are synthesized as latent cytoplasmic precursors with N-terminal regulatory domains. In response to extracellular amino acids the regulatory domains are endoproteolytically excised by the plasma membrane-localized SPS sensor. The processed forms of Stp1 and Stp2 efficiently enter the nucleus and induce expression of amino acid permease genes. We recently reported that the inner nuclear membrane protein Asi1 is required to prevent unprocessed forms of Stp1 and Stp2, which ectopically enter the nucleus, from binding SPS sensor-regulated promoters. Here we show that Asi3, an Asi1 homolog, and Asi2 are integral proteins of the inner nuclear membrane that function in concert with Asi1. In cells lacking any of the three Asi proteins, unprocessed forms of Stp1 and Stp2, which ectopically enter the nucleus, from binding SPS sensor-regulated promoters. Our results demonstrate that the Asi proteins ensure the fidelity of SPS sensor signaling by maintaining the dormant, or repressed state, of gene expression in the absence of inducing signals. This study documents additional components of a novel mechanism controlling transcription in eukaryotic cells.

In eukaryotes the nucleoplasm and cytoplasm are separated by the nuclear envelope. The nuclear envelope consists of two closely aligned bilayers, the inner and outer membranes, each with a unique set of resident proteins. The two nuclear membranes are joined at nuclear pore complexes, which function as channels that provide selective entry and exit routes across the nuclear envelope. Aside from rather detailed knowledge regarding the structure of nuclear pores, there is markedly little known regarding non-pore nuclear proteins (1). However, accumulating data indicate that nuclear envelope proteins participate in a variety of important processes including maintenance of nuclear architecture, chromatin organization, signaling, and gene expression (1–3). Significantly, mutations in genes encoding nuclear envelope proteins are linked to at least 15 inherited human diseases and syndromes (4, 5).

In mammalian cells, nuclear lamins are involved in an extensive network of protein-protein interactions, including inner nuclear membrane proteins and transcriptional regulators, some of which bind DNA (3, 6). Although yeast cells lack lamin homologs (7), processes associated with the inner nuclear membrane have been shown to influence patterns of gene expression. For example, although silencing is not obligatorily linked to perinuclear anchoring (8), recruitment of chromatin to the nuclear periphery can facilitate gene repression (9). Evidence obtained using genome-wide approaches suggest that transcriptionally active regions of chromosomes localize to nuclear pore complexes (10, 11). Consistently, a number of actively expressed genes have been found to interact with the nucleoporin Nup2; the interactions occur at the promoter region of genes and appear to correlate with early events of gene expression (12, 13). Together findings in yeast and metazoan cells suggest that the nuclear envelope, and in particular the inner nuclear membrane, is not only a physical barrier that restricts the movement of macromolecules between the cytoplasm and nucleoplasm, but functions as a scaffold for proteins required for diverse nuclear functions.

Several transcription factors in eukaryotic cells are synthesized as latent precursor forms that are excluded from the nucleus until proper environmental cues activate processes that trigger their targeting and entry into the nucleus (14). Examples of latent transcription factors include the well studied NFκB/Relish, Cubitus interruptus (Ci), and Notch proteins (15–17). The signals that activate and induce the translocation of these factors into the nucleus are initiated by receptors at the plasma membrane. Thus, the mobilization of activated transcription factors physically transmits signals from non-nuclear compartments to specific promoter sequences of responsive genes.

To fully understand how latent factors are controlled, it is crucial to dissect the mechanisms that restrict their activity under non-inducing conditions. Physically anchoring the latent precursor forms outside the nucleus appears to be a versatile mechanism to prevent nuclear import prior to activation. Illustrative examples of this type of negative regulation include the anchoring of the sterol regulatory element-binding protein in membranes of the early secretory pathway (18), and the sequestration of NFκB by the actin cytoskeleton-binding protein IFxB (14, 17). With respect to non-membrane factors like NFκB,
little is known regarding the efficiency of cytoplasmic retention mechanisms. Given the nature and kinetics of protein-protein interactions, retention of soluble latent transcription factors is likely to be incomplete even under non-inducing conditions. Consequently, we anticipate that cells do not merely rely on cytoplasmic retention mechanisms, but possess additional backup systems to maintain the stringency of the inactive state under non-inducing conditions.

In response to extracellular amino acids, the yeast *Saccharomyces cerevisiae* induces the expression of a number of amino acid permeases (AAPs)\(^3\) that collectively control the influx of amino acids across the plasma membrane (19). Induction of AAP gene expression requires the plasma membrane-localized Ssy1-Ptr3-Ssy5 (SPS) sensor (20). The SPS sensor controls the activity of two homologous zinc finger transcription factors Stp1 and Stp2, which are synthesized as latent cytoplasmic factors (21). Upon induction by amino acids, Stp1 and Stp2 are cleaved by the endoprotease Ssy5, a core component of the SPS sensor (22, 23). This liberates the DNA binding and transactivation domains from an ~10-kDa N-terminal regulatory fragment. The shorter forms of Stp1 and Stp2 accumulate in the nucleus and bind to specific upstream activating sequences (UAS\(_{\text{ASi}}\)) within SPS sensor-regulated promoters (24, 25).

To identify regulatory components required to maintain the latent properties of unprocessed Stp1 and Stp2 we isolated recessive mutations in amino acid sensor independent genes that constitutively activate target promoters in the absence of a functional SPS sensor (26). We recently reported that ASI1 encodes an integral polytopic component of the inner nuclear membrane (27). The constitutive expression of SPS sensor-regulated genes in asl\(_{\Delta}\)Δ mutants was traced to the ability of full-length unprocessed forms of Stp1 and Stp2 to enter the nucleus, bind promoters, and induce transcription. Promoter binding of unprocessed forms of Stp1 and Stp2 was not detected in wild-type cells. These findings provided evidence of the involvement of inner nuclear membrane proteins in an unanticipated mechanism of transcriptional control. Here we report that ASI2 and ASI3 also encode integral components of the inner nuclear membrane. Our results indicate that Asi2 and Asi3 function together with Asi1 to prevent the unprocessed forms of Stp1 and Stp2 that escape cytoplasmic retention mechanisms from inducing SPS sensor-controlled gene expression. These findings demonstrate that the Asi proteins ensure the fidelity of SPS sensor signaling by maintaining the dormant, or repressed state of gene expression in the absence of inducing signals.

**EXPERIMENTAL PROCEDURES**

**Media, Strains, and Plasmids**—Standard media including YPD and ammonia-based synthetic minimal dextrose (SD), supplemented as required to enable growth of auxotrophic strains, were prepared as described (28). Ammonia-based synthetic complex dextrose (SC) was prepared as described (21). Where indicated, l-leucine was added at a concentration of 1.3 mM to induce the SPS sensor. When required 5-fluoroorotic acid (1 g/liter) was added to SC. Media were made solid with 2% (w/v) BactoAgar (Difco). Antibiotic selections were made on solid YPD supplemented with 200 mg/liter G418 (Invitrogen), 100 mg/liter clonNAT (Werner Bioagents, Jena, Germany), or 300 mg/liter hygromycin B (Duchefa, Haarlem, Netherlands). Sensitivity to 1 mM l-azetidine-2-carboxylic acid (AzC) was tested on SD supplemented with l-leucine (1.3 mM) and l-glutamic acid (1 mM). Sensitivity to 2-[[4-(methoxy-6-methyl)-1,3,5-triazin-2-yl]-amino]carbonyl]amino][sulfonfyl]-benzoic acid (MM) was tested on YPD at a concentration of 500 mg/liter (29). The yeast strains used are listed in Table 1. All strains are isogenic descendents of the S288c-derived strain AA255/PLY115 (30). Plasmids used are listed in Table 2. Details regarding strain and plasmid constructions are provided online in supplementary materials.

**Northern Analysis**—Total RNA was prepared using the hot phenol method from cells growing in SD or SD supplemented with leucine. After separation of RNA on a denaturing agarose gel, RNA was transferred to a nylon membrane (Hybond-N\(^+\); Amersham Biosciences). Digoxigenin-labeled probes, specific for 339-bp AGP1 fragment (20) and 1.65-kb ACT1 fragment (31) were generated by PCR. Probes were hybridized and blots were developed according to the manufacturer’s instructions (Roche). Results were quantified using an LAS1000 system (Fuji Photo Film Co. Ltd.).

**β-Galactosidase Activity Assay**—β-Galactosidase activity was determined with N-lauroylsarcosine-permeabilized cells (32). Cells grown in SD were harvested by centrifugation, resuspended in Z buffer, and the A\(_{600}\) was measured. A 250-μl aliquot of cell suspension was mixed with 550 μl of 0.3% (w/v) sodium N-lauroylsarcosine Z-buffer and incubated at 30 °C for 15 min. A 160-μl aliquot of 2-nitrophenyl β-galactopyranoside solution (4 mg/ml) was added and tubes were mixed by vortexing. The reactions were stopped by the addition of 400 μl of 1 M Na\(_2\)CO\(_3\), the tubes were centrifuged at 12,000 × g for 5 min, and the absorbance of the supernatant measured at 420 nm. Activity was calculated according to formula: 10\(^3\) × A\(_{420}/A_{600}\)/0.25 (volume of cell suspension added)/time (min).

**Stp1 and Stp2 Processing**—Cells were grown in amino acid-free SD medium to an A\(_{600}\) of 0.8–1.0, cultures were split, and cells were harvested 30 min after the addition of an aliquot of l-leucine (final concentration 1.3 mM) or water. Extracts were prepared from 1 ml of cell cultures according to Ref. 33. Extracted proteins were resolved on 10% acrylamide gel using SDS-PAGE and analyzed by immunoblotting. Immunoblots were incubated with primary antibody (12CA5 ascites fluid; anti-HA monoclonal) diluted 1:1000 in blocking buffer (PBS, pH 7.4, 0.1% Tween 20, 5%, low fat milk powder). Immunoreactive bands were visualized by chemiluminescence detection (SuperSignal® West Dura Substrate; Pierce) of horseradish peroxidase conjugated to a secondary antibody (anti-mouse Ig from sheep, and anti-rabbit Ig from donkey; Amersham Bio-

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\(^3\) The abbreviations used are: AAP, amino acid permease; SPS, Ssy1-Ptr3-Ssy5; SD, synthetic minimal dextrose; SC, synthetic complex dextrose; AzC, azetidine carboxylate; MM, 2-[[4-(methoxy-6-methyl)-1,3,5-triazin-2-yl]-amino]carbonyl]amino][sulfonfyl]-benzoic acid; PBS, phosphate-buffered saline; endo-H, endoglycosidase H; HA, hemagglutinin; BSA, bovine serum albumin; PPS, phosphate-buffered saline; SLS, sodium N-lauroyl sarcosinate; GST, glutathione S-transferase; ER, endoplasmic reticulum; MOPS, 4-morpholinepropanesulfonic acid; WT, wild type.
TABLE 1
Yeast strains

| Strain        | Genotype                              | Ref. |
|---------------|---------------------------------------|------|
| A2Y282        | MATa ura3-52 ASI3::13MYC               | 27   |
| CAY28         | MATa ura3-52                           | 21   |
| CAY29         | MATa ura3-52                           | 21   |
| CAY92         | MATa ade2 len2-3, 112 lys2Δ201 ura3-52 sst1Δ13 ASI3-3HA::URA3::3HA This study |      |
| CAY93         | MATa ade2 len2-3, 112 lys2Δ201 ura3-52 sst1Δ13 ASI3-3HA This study |      |
| MBY21         | MATa ura3-52 trplΔ101::ASD-6HA::KTRP1  | This study |      |
| MBY23         | MATa ura3-52 sst1Δ90::natMX4 asi2Δ9::hisG::URA3::asi3Δ5::kanMX4 This study |      |
| MBY26         | MATa ura3-52 sst1Δ51::Aglen2 trp2Δ50::hphMX4 asi2Δ9::hisG::URA3::hisG This study |      |
| MBY28         | MATa ura3-52 sst1Δ51::Aglen2 trp2Δ50::hphMX4 asi2Δ90::natMX4 asi2Δ9::hisG asi3Δ5::kanMX4 This study |      |
| MBY39         | MATa ura3-52 sst1Δ77::natMX4            | This study |      |
| MBY106        | MATa ura3-52 sst1Δ77::natMX4 asi1Δ80::hphMX4 This study |      |
| MBY108        | MATa ura3-52 sst1Δ77::natMX4 asi2Δ9::hisG This study |      |
| MBY110        | MATa ura3-52 sst1Δ77::natMX4 asi3Δ5::kanMX4 This study |      |
| MBY112        | MATa ura3-52 sst1Δ77::natMX4 asi1Δ80::hphMX4 asi2Δ9::hisG This study |      |
| MBY114        | MATa ura3-52 sst1Δ77::natMX4 asi1Δ80::hphMX4 asi3Δ5::kanMX4 This study |      |
| MBY116        | MATa ura3-52 sst1Δ77::natMX4 asi2Δ9::hisG asi3Δ5::kanMX4 This study |      |
| MBY118        | MATa ura3-52 sst1Δ77::natMX4 asi1Δ80::hphMX4 asi2Δ9::hisG asi3Δ5::kanMX4 This study |      |
| PLY860        | MATa ura3-52 trp1Δ101::lexP             | This study |      |
| PLY1314       | MATa ura3-52 sst1Δ80::hphMX4            | 27   |
| PLY1315       | MATa ura3-52 asi2Δ9::hisG::URA3::hisG  | This study |      |
| PLY1318       | MATa ura3-52 asi3Δ5::kanMX4            | This study |      |
| PLY1322       | MATa ura3-52 asi1Δ80::hphMX4 asi3Δ5::kanMX4 This study |      |
| PLY1326       | MATa ura3-52 asi1Δ80::hphMX4 asi2Δ9::hisG::URA3::asi3Δ5::kanMX4 This study |      |
| PLY1340       | MATa ura3-52 asi2Δ9::hisG              | This study |      |
| PLY1341       | MATa ura3-52 asi2Δ9::hisG              | This study |      |
| PLY1343       | MATa ura3-52 asi1Δ80::hphMX4 asi2Δ9::hisG This study |      |
| PLY1345       | MATa ura3-52 asi2Δ9::hisG::URA3::asi3Δ5::kanMX4 This study |      |
| PLY1346       | MATa ura3-52 asi1Δ80::hphMX4 asi2Δ9::hisG asi3Δ5::kanMX4 This study |      |
| PLY1347       | MATa ura3-52 asi1Δ80::hphMX4 asi2Δ9::hisG asi3Δ5::kanMX4 This study |      |
| YMHS45        | MATa ura3-52 trplΔ101::lexP             | This study |      |
| YMHS49        | MATa ura3-52 sst1Δ77::natMX4            | 26   |

TABLE 2
Plasmids

| Plasmid       | Description                              | Ref. |
|---------------|------------------------------------------|------|
| pA2002        | ASI1-3HA in pRS316                       | 27   |
| pA2014        | ASI1-3HA in pRS202                       | 27   |
| pA2017        | ASI1(4A)-3HA in pRS316                  | This study |      |
| pA2018        | ASI1(21A)-3HA in pRS316                 | This study |      |
| pA2019        | ASI1(21A,290)-3HA in pRS316             | This study |      |
| pA2020        | ASI1(4A,21A,290)-3HA in pRS316          | 27   |
| pA2042        | ASI2-3HA in pRS316                      | This study |      |
| pA2043-054    | BglII sites introduced into ASI2 in pAZ042 | This study |      |
| pA2055-066    | suc2 topology reporter inserted into BglII sites in pAZ042-054 | This study |      |
| pA2067        | GST-ASI1-3HA in EGK                     | This study |      |
| pCA042        | STP1 in pRS316                          | 21   |
| pCA047        | STP1-3HA in pRS316                      | 21   |
| pCA111        | STP2-3HA in pRS316                      | 21   |
| pCA207        | PGPN1-1acz in CEN URA3                  | 27   |
| pEGKT         | GST in 2-µL URA3                        | 55   |
| pMB3          | ASI2-3HA in pRS202                      | This study |      |
| pMH5          | ASI2 in pRS316                          | This study |      |
| pMB55         | ASI2-13MYC in pRS316                    | This study |      |
| pRS202        | 2-µL URA3                               | 56   |
| pRS316        | CEN URA3                                | 57   |
| YCpAGP1-LACZ  | PAGP1-1acz in CEN URA3                  | 58   |

Microscopy—Cells were grown to an A600 of 0.8 and processed for indirect immunofluorescence analysis essentially as described in Ref. 28. Cells were fixed by the addition of an aliquot of 37% formaldehyde directly to the cultures to a final concentration of 4.5%, and incubated 45 min at 30 °C. To detect HA- and myc-tagged proteins, the 12CA5 and 9E10 monoclonal antibodies diluted 1:300 were used, respectively. The secondary antibody was Alexa Fluor 488 conjugated to goat anti-mouse (H + L; Molecular Probes), diluted 1:500. Cells were viewed using a Zeiss Axioskop microscope with a Plan-Apochromat ×63/1.40 objective. Digital images of cells were examined using Nomarski optics, and antibody dependent and 4',6-diamidino-2-phenylindole fluorescence (standard filter sets), were captured using a C4742-95 CCD camera (Hamamatsu Photonics, Japan) and QED Imaging software (Media Cybernetics). Image files were incorporated into figures using Photoshop CS (Adobe Systems, Inc.).

Immunoelectron Microscopy—Cells were grown to an A600 of 0.7 and fixed in 2% formaldehyde, 0.1% glutaraldehyde at 30 °C for 1 h with gentle shaking. The fixed cells were washed in SP buffer (1.2 m sorbitol, 0.1 m potassium phosphate, pH 7.5), pelleted by centrifugation, dehydrated in graded ethanol (70–100%), and embedded in LR White Resin (London Resin) by UV polymerization as described (27). Thin sections were cut on a Leica Ultracut and picked up on formvar-coated nickel grids. For immunostaining, the sections were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.3, for 30 min, and incubated for 120 min with rat anti-HA antibodies (clone 3F10, Roche) diluted 1:50 in PBS, 5% BSA. The grids were then rinsed repeatedly with PBS, 5% BSA and incubated for 60 min with goat anti-rat IgG conjugated to 12-nm gold particles (Jackson ImmunoResearch) diluted 1:20.

sciences), and quantified by using the LAS1000 system (Fuji Photo Film Co. Ltd.).

Determination of Glycosylation Status and Membrane Topology—Whole cell extracts derived from 1 ml of cultures (A600 of 1) were prepared (33). Duplicate protein samples (25 µL, equivalent to A600 of 0.2 cell suspension) were diluted with an equal volume of 100 mM sodium citrate, pH 5.5, and heated for 10 min at 37 °C. Three milliliters of endoglycosidase H (endo-H) (Roche) were added to half of the samples, and all samples were incubated overnight at 37 °C (34). Proteins were resolved by 7% SDS-PAGE and immunoblotted with monoclonal anti-HA antibody (12CA5).
in PBS, 0.5% BSA. After repeated washing with PBS, 0.5% BSA and PBS alone, the grids were postfixed for 5 min with 2% glutaraldehyde in PBS, washed with PBS and water, and allowed to dry. Contrast staining was made with saturated uranyl acetate for 5 min and lead citrate for 5 s. Finally, the sections were examined in a Philips CM120 electron microscope at 80 kV and photographed using a CCD camera (Kodak MegaPlus). For quantitative evaluation of the immunogold labeling, successive cells were photographed at high magnification and the distance of all gold particles from the midline of the nuclear envelope was measured using the analySIS system (Soft Imaging Software, Münster, Germany).

Subcellular Fractionation—Cells expressing functional epitope-tagged ASI1-HA, ASI2-HA, and ASI3-HA were grown in SC to an A600 of 0.8. Cells were harvested by centrifugation, and protein extracts were prepared by vortexing cells seven cycles (30 s vortex followed by 30 s on ice) with glass beads (1 µm in diameter) in lysis buffer (0.8 M sorbitol, 10 mM MOPS, pH 7.2, 2 mM NaEDTA) containing 1 mM phenylmethylsulfonyl fluoride, 10 units/ml Trasylol, and Complete protease inhibitors (Roche). Unbroken cells were removed by centrifugation at 1000 × g for 5 min at 4 °C. The extracted proteins were fractionated on 12–60% sucrose gradients as described by Ref. 35. Fractions (1 ml) were collected from the bottom of the gradients using a Fraction Recovery System (Beckman). Proteins from equal aliquots of fractions were concentrated by trichloroacetic acid precipitation, separated by SDS-PAGE, and blotted onto nitrocellulose membranes. Blots were incubated with primary antibodies in blocking buffer diluted as follows: 12CA5 ascites fluid (Anti-HA monoclonal), 1:1500; rabbit anti-Pma1p, 1:1500; mouse monoclonal anti-Vps10p, 1:1000.

ASI1, ASI2, and ASI3 Membrane Association—Cells, grown in SC to an A600 of 0.8, were harvested by centrifugation, washed once with cold 10 mM NaN3, flash frozen in liquid nitrogen, and stored at −80 °C. Cell lysates were prepared and fractionated on 12–60% sucrose gradients as described in the preceding section. Microsomal fractions containing Asi proteins (fractions 1–6, from the bottom) were pooled and diluted 1:2 with HDB buffer (25 mM HEPES, 0.7 mM Na2HPO4, 137 mM NaCl, 5 mM KCl, pH 7.3–7.4) containing Complete protease inhibitor (Roche). Membranes were isolated by centrifugation at 100,000 × g for 1.5 h at 4 °C. Membrane pellets were resuspended in 200 µl of HDB buffer containing Complete protease inhibitor, and the protein concentration was measured (Bio-Rad). Aliquots of membrane suspensions (600 µg) were incubated in the presence of detergent for 15 min at room temperature; samples were continuously mixed by inversion. Attempted solubilization conditions are as follows: 1–10% N-dodecylmaltoside (Roche Molecular Biochemicals); 1% N-dodecylmaltoside containing 70, 140, or 280 mM NaCl; 1% Triton X-100 (Sigma); 1% Tween 20 (Sigma); 0.5% Nonidet P-40 (Sigma); 1–2% sodium N-lauroyl sarcosinate (SLS) (Sigma); or 1% SLS and 1% Triton X-100. Detergent extracts were centrifuged at 100,000 × g for 1.5 h at 4 °C. Proteins in soluble and pellet fractions were resolved by SDS-PAGE in 10% polyacrylamide gels, and immunoblotted. Blots were incubated with primary antibodies in blocking buffer diluted as follows: 12CA5 ascites fluid (anti-HA monoclonal), 1:1500; anti-myc (monoclonal antibody 9E10), 1:1500; and anti-Dpm1 (monoclonal antibody), 1:500.

Immunoprecipitation and Glutathione S-Transferase (GST) Purifications—Cells, pregrown in SC and washed once with sterile water, were used to inoculate SC containing 8% galactose (no glucose) to an initial A600 of 0.05, and cultures were grown to an A600 of 0.8. Cells were harvested, washed once in cold 10 mM NaN3, and flash frozen in liquid nitrogen. Microsomes containing Asi proteins were prepared and membrane proteins were solubilized in the presence of 1% SLS and 1% Triton X-100. Detergent-solubilized proteins were incubated overnight at 4 °C with glutathione-Sepharose 4B beads (Amersham Biosciences) or anti-HA affinity matrix (Roche). The beads were pelleted by centrifugation for 2 min at 3,000 × g (supernatant = flow-through) and washed five times with HDB buffer containing 0.1% Triton X-100 and Complete protease inhibitor. Twenty µl of 2× SDS sample buffer were added to 20 µl of the beads to elute bound protein. Proteins, denatured for 10 min at 45 °C, were resolved by SDS-PAGE in 12% polyacrylamide gels, and immunoblotted. Anti-GST rabbit polyclonal antibody was used at a concentration of 2 µg/ml.

Online Supplemental Material—Detailed descriptions of strain and plasmid constructions are described in the supplemental materials.

RESULTS

Asi2 and Asi3 Function Similar to Asi1 as Components of the SPS Sensor Regulon

Null mutations in ASI1, ASI2, and ASI3 are recessive, and lead to constitutive transcription of SPS sensor-regulated amino acid permease genes (19), indicating that Asi proteins function as negative regulators of SPS sensor signaling. We have shown that Asi1 negatively regulates gene expression under non-inducing conditions, i.e. in the absence of extracellular amino acids. It does so by preventing transcription factors Stp1 and Stp2 from binding SPS sensor-regulated promoters (27). We anticipated that if the Asi2 and Asi3 proteins function similarly to Asi1, then strains carrying multiple mutations in ASI genes would display phenotypes similar to those of the single mutant strains. Also, the constitutive expression of SPS sensor-regulated genes in asi2 and asi3 mutants would be Stp1 and Stp2 dependent. Conversely, if the Asi proteins function independently, strains carrying multiple mutations would likely exhibit altered or exaggerated phenotypes. Furthermore, the constitutive expression of SPS sensor-regulated genes could be Stp1 and Stp2 independent.

We constructed strains carrying individual and all possible combinations of null mutations in the ASI genes, and monitored the level of the SPS sensor-regulated AGP1 expression. In wild-type cells grown in the absence of amino acids, AGP1 expression is low. However, 30 min after the addition of 1.3 mM leucine, AGP1 expression is induced ~10-fold (Fig. 1A, compare lanes 1 and 2). AGP1 expression is constitutive in strains carrying asi1, asi2, or asi3 null alleles (Fig. 1B, lanes 3–5). The level of AGP1 expression in the mutants is similar to the level of amino acid-induced expression in wild-type cells. The strains
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FIGURE 1. Characterization of the SPS sensor signaling pathway in asi null mutant strains. A, the expression of AGP1 was assessed in wild-type (WT), CAS1 (PLY1314), asiΔ (PLY1341), asi2Δ (PLY1341), asiΔ asi2Δ (PLY1343), asiΔ asi3Δ (PLY1322), asi2Δ asi3Δ (PLY1345), and asiΔ asi2Δ asi3Δ (PLY1347) strains. The strains were grown in SD (−) or SD supplemented with leucine (+). Levels of AGP1 are normalized to ACT1 in each sample, and to induced levels present in wild-type cells (lane 2). B, loss-of-function mutations in ASI genes derepress SPS sensor gene expression in a Stp1- and Stp2-dependent manner. Expression of AGP1 (left panel) and GNPI (right panel) was monitored by measuring β-galactosidase activity of PAGP1-lacZ and PGNP1-lacZ reporter constructs. Strains CAY29 (WT), PLY1346 (asiΔ asi2Δ asi3Δ = asi1-3Δ), and MBY028 (asi1-3 stp1 stp2), carrying plasmids YCPAGP1-lacZ (PAGP1-lacZ) or pCA227 (PGNP1-lacZ) were grown in SD (−leu) or SD supplemented with leucine (+leu). β-Galactosidase activity present in three independent transformants of each strain was quantified; error bars indicate 1 S.D.

carrying multiple asi mutations exhibited similar levels of AGP1 expression as single mutant strains (Fig. 1A, lanes 6–9). The lack of differential effects in strains with multiple mutations is consistent with the idea that the Asi proteins function in the same regulatory step.

Next, we examined whether the constitutive expression of SPS-sensor genes in the triple asiΔ asi2Δ asi3Δ mutant strain (asi1-3) was Stp1 and Stp2 dependent. The levels of AGP1 and GNPI expression were measured in wild-type (WT), asiΔ-3, and asi1-3 stp1Δ stp2Δ strains carrying either PAGP1-lacZ (Fig. 1B, left panel) or PGNP1-lacZ (Fig. 1B, right panel) reporter constructs. Cells were grown in the presence or absence of the inducing amino acid leucine. In wild-type cells the expression of β-galactosidase was dependent upon amino acid availability; low levels of β-galactosidase were detected in uninduced cells (SD), whereas robust activity was detected upon induction (SD + Leu). Constitutive expression of both reporter constructs was observed in the asi1Δasi1-3 mutant. Importantly, β-galactosidase activity was not detected in the asi1Δasi2Δ asi2Δ mutant, thus the constitutive transcription of SPS sensor-regulated genes in asi mutants is strictly dependent on the presence of Stp1 or Stp2. These results demonstrate that Asi2 and Asi3 function similarly to Asi1 as components of the SPS-signaling pathway that negatively modulate the activity of Stp1 and Stp2 under non-inducing conditions.

asi Mutations Enable Signaling by Unprocessed Forms of Stp1 and Stp2

The N-terminal regulatory domains of Stp1 and Stp2 are modular and transferable, and confer SPS sensor pathway control when fused to unrelated synthetic transcription factors (36). The N-terminal domains possess two conserved sequence motifs, designated Regions I and II. Two parallel activities converge on Region I (27). The primary activity serves to anchor the unprocessed full-length forms to an undefined, cytoplasmic determinant, which prevents targeting of unprocessed factors to the nucleus. The second, Asi1-dependent, activity prevents the low levels of full-length Stp1 and Stp2 that inappropriately enter the nucleus from activating transcription. Region II mediates SPS sensor-dependent endoproteolytic processing, and thus represents the third regulatory activity that controls the transactivation potential of Stp1 and Stp2 (23). We tested whether Asi2 and Asi3 affect one of the three regulatory activities impinging on the N-terminal regulatory motifs of Stp1 and Stp2.

We first investigated the possibility that Asi2 or Asi3 function by affecting regulatory motif Region II-mediated processing and prevent Stp1 and Stp2 processing under non-inducing conditions. If this was the case, then Stp1 and Stp2 should be constitutively processed in asi2Δ asi3Δ mutants. Stp1 and Stp2 processing was examined in wild-type and asi1Δasi3Δ strains (Fig. 2A). In the absence of amino acids, Stp1 and Stp2 were found exclusively in their unprocessed full-length forms (lanes 1–4), and both factors were processed normally in amino acid-induced cells (lanes 5–8). Thus, similar to Asi1 (27), Asi2 and Asi3 do not appear to affect Stp1 and Stp2 processing. Together with our finding that constitutive transcription in asi2Δ and asi3Δ mutants is strictly Stp1 and Stp2 dependent (Fig. 1B), the data strongly suggest that the loss of Asi2 or Asi3 function enables unprocessed full-length forms of Stp1 and Stp2 to induce SPS sensor-regulated genes.

To eliminate the possibility that asi2Δ and asi3Δ mutations affect processing at levels too low to detect by immunoblot analysis, we tested whether the constitutive activation of SPS sensor-regulated genes is still present in asi2Δ and asi3Δ strains lacking the Stp1 and Stp2 processing endoprotease Ssy5 (Fig. 2B). SPS sensor-dependent expression of amino acid permease genes can readily be assessed in growth-based assays in which growth depends on the SPS sensor-regulated uptake of amino acids or toxic amino acid analogues. The first growth-based assay examines the ability of cells to take up branched chain amino acids in the presence of high concentrations of competing amino acids; growth is scored on rich YPD medium supplemented with MM, an inhibitor of branched chain amino acid synthesis. On this medium, branched chain amino acid uptake
INM Proteins Asi1, Asi2, and Asi3 Function in Concert

An intriguing aspect regarding Asi1, Asi2, and Asi3 is that each of these proteins has multiple hydrophobic stretches that are predicted to be membrane-spanning segments. Understanding how these integral membrane proteins negatively modulate SPS sensor-regulated gene expression necessitates detailed knowledge of their membrane structure and topology.

Asi3 and Asi1 Are Homologous—In direct comparisons, Asi3 (669 amino acids) and Asi1 (624 amino acids) are 31% identical and 42% similar. With the exception of minor gaps, the homology is distributed over the entire length of their amino acid sequences (Fig. 3A). Both proteins have two equally sized domains: an N-terminal domain with five hydrophobic segments, and a hydrophilic C-terminal domain ending with a highly conserved Zn$^{2+}$ binding RING motif (26). We have recently shown that Asi1 has an odd number of membrane spanning segments with the N- and C-terminal domains oriented toward opposite sides of the membrane (Fig. 3B, right panel) (27). It is likely that Asi3 has a similar structure. However, despite the high degree of homology, we have found that plasms containing inserts encoding Asi3 and Asi1 are differentially tolerated when passed through Escherichia coli. Whereas plasms containing Asi1 are propagated without affecting growth, plasms with DNA fragments spanning the Asi3 open reading frame, and including as little as 279 bp 5′- and 102 bp 3′-sequences, impair growth, and are not stably maintained. Furthermore, recovered plasms often contain grossly rearranged inserts, suggesting that expression of Asi3 is deleterious. Consequently, to compare the structural similarity between Asi1 and Asi3, a functional HA epitope-tagged allele of Asi3 was obtained by homologous recombination in yeast.

Asi3 has two possible N-glycosylation sites, one situated in the N terminus prior to the first membrane-spanning segment and the other located in the C-terminal domain (Fig. 3B, left panel). The Asi1 sequence contains 11 possible N-glycosylation sites, three of which are located in the N-terminal domain preceding the first membrane-spanning segment (Fig. 3B, right panel).
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**FIGURE 3. Comparative analysis of Asi3 and Asi1.** A, Asi3 and Asi1 are homologous proteins. Schematic presentation of alignment of Asi3 (green, 669 amino acids) and Asi1 (blue, 624 amino acids) using the GAP algorithm (GCG Wisconsin sequence Analysis Package) in accordance to the BLOSUM62 amino acid substitution matrix. The gap and length weight parameter settings used were 8 and 2, respectively. The positions of identical amino acid residues are indicated by black bars, and the location of five segments predicted to function as membrane spanning domains are indicated (red bars, I-V) (54). B, Asi3 and Asi1 are glycoproteins with luminal oriented N-terminal domains. Schematic presentation of the predicted Asi3 (left panel) and experimentally determined membrane topology of Asi1 (right panel); the colored boxes depict the five hydrophobic domains (I-V), the C-terminal RING domains and the HA epitope tags are indicated. The positions of possible N-linked glycosylation sites are shown. The glycosylation status of Asi3-HA and Asi1-HA was determined (middle panel). Strains expressing Asi3-HA (CAY93) and Asi1-HA (YMH349(pAZ002)) were grown in SC, cell extracts were prepared, and treated with (+) or without (−) endo-H. Proteins were resolved on 10% SDS-PAGE gels and blotted with anti-HA antibody. C and D, glycosylation is not required for Asi1 function. C, immunoblot analysis of extracts from strain YMH349 (ssy1 Δ leu2 asi1 Δ) expressing wild-type Asi1-HA (WT, N N N, pAZ002) or mutant forms of Asi1-HA lacking N-terminal N-linked glycosylation sites as indicated, i.e., 2 (4A, X N N, pAZ017), 19 (21A, X N N, pAZ018), 19, 21 (21A,29Q, N X X, pAZ019), and 2, 19, and 29 (4A,21A,29Q, X X X, pAZ020). Extracts were analyzed as in B, 10-fold dilutions of cell suspensions of strains as in C and YMH349 carrying pRS316 (vector) were spotted onto plates containing SD or SC, incubated for 3 days at 30°C and photographed. CHO, Chinese hamster ovary.

panel). The glycosylation state of Asi3-HA and Asi1-HA was analyzed by treatment with endo-H (Fig. 3B, middle panel). A clear increase in mobility was observed after endo-H treatment demonstrating that Asi3 and Asi1 are glycoproteins. Due to the difficulty of manipulating cloned DNA fragments containing Asi3, we pursued the biological significance of glycosylation by introducing single amino acid changes that individually abolished the glycosylation sites in the N terminus of Asi1; each mutation lowered the extent of glycosylation as, evidenced by an increased rate of migration (Fig. 3C, compare lanes 3, 5, and 6 with lane 1), clearly indicating that the N terminus is oriented toward the ER lumen during biogenesis. The simultaneous mutation of all three sites eliminated glycosylation in Asi1 (Fig. 3C, lane 9), indicating that the other potential glycosylation sites present in Asi1 are not used, which is consistent with an extraluminal orientation.

We examined the biological significance of Asi1 glycosylation using a growth-based assay. In short, leucine auxotrophic (leu2) cells lacking a functional SPS sensor (ssy1) are unable to grow on SC medium (26). The introduction of an asi1 Δ null allele into ssy1 leu2 strains restores growth on SC (Fig. 3D, dilution series 1, vector). The recessive nature of asi1 mutations enables plasmids encoding functional ASI1 constructs to complement the mutant phenotype, consequently, cells do not grow on SC (Fig. 3D, dilution series 2 and 3). Similarly, strains carrying the glycosylation defective mutant alleles of ASI1, i.e., 4A, 21A, 21A29Q, and 4A,2A,29Q (CHO−) (Fig. 3D, dilution series 4–7) did not grow, indicating that glycosylation is not required for Asi1 function.

Asi2 Has Two Membrane-spanning Segments—Asi2 has an hydrophilic N-terminal domain and three hydrophobic segments that are predicted to be membrane spanning (Fig. 4A). Asi2 has two potential N-glycosylation sites at amino acids 11 and 67, however, treatment with endo-H did not affect its electrophoretic mobility, indicating that Asi2 is not a glycoprotein (Fig. 4B, compare lanes 1 and 2). The lack of endogenous glycosylation enabled us to assess Asi2 structure by inserting a topological reporter cassette (34) at diagnostic positions within the hydrophilic N-terminal domain, in the two hydrophilic loops (L1 and L2) that separate the three hydrophobic segments (I–III), and in the C-terminal domain (Fig. 4A). The resulting gene-sandwich fusions encode func-
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The results indicating that Asi2 and Asi3 function similarly to Asi1 (Figs. 1 and 2), prompted us to investigate whether Asi3 and Asi2 co-localize with Asi1 to the inner nuclear membrane (27). The intracellular location of functional Asi2-myc and Asi3-myc epitope-tagged proteins was examined by immunofluorescence microscopy (Fig. 5A). Both Asi2-myc and Asi3-myc fluorescence was restricted to and evenly distributed in the nuclear envelope (Fig. 5, lower and upper panels, respectively). The pattern of fluorescence was indistinguishable from the continuous rim staining of Asi1-HA (middle panel). Asi2 remained evenly distributed in the nuclear membrane of *nup133Δ* mutant cells (data not shown). In this mutant, nuclear pore-associated proteins cluster to distinct areas of the nuclear envelope resulting in a single intense spot of fluorescence when cells are stained with anti-nuclear pore antibodies (38, 39). Thus, similar to Asi1 (27), Asi2 is not intimately associated with nuclear pores.

To more precisely localize Asi2 and Asi3 we used post-embedding immunoelectron microscopy, as described in Ref. 27 (Fig. 5B). In this method, the nuclear envelope appears in negative contrast as a light band surrounding the nucleus, which includes both the outer and inner nuclear membranes. We quantified the number of immunogold particles associated with different compartments in the cell (Table 3); for comparison purposes, previously published data regarding Asi1 are included (27). The major portion of the Asi2- and Asi3-dependent immunogold particle labeling was found associated with the nuclear compartment. Thus Asi1 (27), Asi2, and Asi3 are components of the inner nuclear membrane.

**Biochemical Characterization of Asi1, Asi2, and Asi3**

Our genetic analysis suggested that the Asi proteins likely function in concert to regulate SPS sensor gene expression (Figs. 1 and 2). Based on our finding that the Asi proteins are integral membrane proteins that co-localize to the inner nuclear membrane, we considered the possibility that they function together within a complex. We set up a series of biochemical tests to evaluate this possibility. If the Asi proteins are indeed components of a complex then they should co-fractionate, exhibit similar solubility in detergents, and co-purify.

**Asi1, Asi2, and Asi3 Co-fractionate**

*Cell lysates prepared from strains expressing functional HA-tagged alleles of ASI1, ASI2, and ASI3 were fractionated on 12–60% step sucrose gradients. The proteins in each fraction were resolved by SDS-PAGE and analyzed on immunoblots incubated with antibodies specific for organelle-specific marker proteins and the HA tag (Fig. 6). In each instance, the HA-tagged proteins co-fractionated with the ER marker protein Wbp1. The finding that all three Asi proteins fractionate similarly is consistent with our microscopic analysis. Apparently, the inner nuclear membrane of yeast has a similar density as the ER membrane, which is continuous with the outer nuclear membrane.*

**Detergent Solubility of Asi1 and Asi3**

*We evaluated the ability of various detergents to solubilize Asi1 and Asi3 from...*
isolated microsomal/nuclear membranes. Consistent with being integral nuclear membrane proteins, the Asi proteins were not solubilized in the presence of non-ionic detergents, even when the salt concentration was raised to 280 mM NaCl. In contrast, the anionic detergent SLS (40) was able to liberate both Asi1 and Asi3 from the membrane fraction (Fig. 7A). In the presence of 1% SLS, almost all Asi3 and the major portion of Asi1 was recovered in the soluble fraction. Increasing the concentration of SLS from 1 to 2% decreased the efficiency of extraction. We found that by combining 1% SLS with 1% Triton X-100 the remaining Asi1 and Asi3 could be solubilized (Fig. 7A, compare lane 6 with 8). Under these conditions, 50% of Asi2 was solubilized from membranes (data not shown). The Asi proteins were not solubilized in the presence of the non-ionic detergents N-dodecylmaltoside (1–10% /H11006 70–280 mM NaCl), Triton X-100 (1%), Tween 20 (1%), or Nonidet P-40 (1%) (data not shown). The control ER membrane protein Dpm1 was efficiently released from the membranes in the presence of all detergents tested and was always recovered in the soluble fraction.

Asi1 and Asi3 Co-purify—The ability to simultaneously solubilize both Asi1 and Asi3 encouraged us to evaluate whether these two proteins co-purify. Microsomal/nuclear membranes were isolated from strains expressing ASI3-myc and a functional GST-tagged allele of ASI1-HA (GST-ASI1-HA). A strain expressing ASI3-myc and only GST was included as a control. Membranes were solubilized in the presence of 1% SLS and 1% Triton X-100, and the levels of Asi1 and Asi3 in the soluble fractions were analyzed (Fig. 7B, lane 1). GST-Asi1-HA in soluble fractions was purified using glutathione-Sepharose 4B or immunoprecipitated with anti-HA affinity matrix. After extensive washing, we found that Asi3-myc specifically associated with purified Asi1 (Fig. 7B, lanes 4, 6, and 8) but not with the purified GST control (Fig. 7C). The control protein Dpm1 did not co-purify or co-immunoprecipitate (Fig. 7B). To summarize, these biochemical approaches demonstrate that Asi1 and Asi3 co-purify and suggest that these proteins function as a part of a complex.

FIGURE 5. Asi3 and Asi2 are components of the inner nuclear membrane. A, indirect immunolocalization of Asi3, Asi1, and Asi2 was performed with anti-myc (α-myc) or anti-HA (α-HA) antibodies. Strain AZY262 expressing Asi3-myc was grown in YPD, and strains expressing Asi1-HA (PLY1314(pAZ014)) and Asi2-myc (PLY1340(pMB55)) were grown in SC (−ura). Panels, left to right: antibody (α-myc or α-HA)-dependent Alexa Fluor 488 fluorescence; 4',6-diamidino-2-phenylindole (DAPI) staining; cells viewed by Nomarski optics. The bar in the lower left panel corresponds to 5 μm. B, immunoelectron microscopic analysis of Asi3, Asi1, and Asi2 localization. Strains expressing Asi3-HA (MBY21), Asi1-HA (PLY1314(pAZ014)), or Asi2-HA (PLY1341(pMB3)) were grown in SC (−ura) to an A600 of 0.7. Cells were fixed and prepared for analysis as described under “Experimental Procedures.” Immunoelectron micrographs of yeast cell nuclei (Nu)(left panels). The white arrowheads indicate the nuclear envelope (NE) and the black arrowheads show the location of the HA-epitope dependent labeling of immunogold particles (IGP). Scale bar corresponds to 0.5 μm. Quantitative analysis of the distribution of intracellular immunogold labeling (right panels); the distances between the IGP and the nuclear envelope are plotted.
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DISCUSSION

The SPS-sensing pathway enables yeast cells to respond to the presence of extracellular amino acids and promote their uptake. The activating step of the SPS-sensing pathway is the endoproteolytic processing of Stp1 and Stp2, two latently expressed transcription factors (21, 23). In the absence of inducing amino acids, these factors are restricted from the nucleus due to N-terminal cytoplasmic retention motifs. We have recently obtained evidence suggesting that cytoplasmic retention is not absolute, and that a backup system prevents the unprocessed forms of these factors that escape cytoplasmic anchoring mechanisms from binding to SPS sensor-regulated promoters (27). In this article we document that this backup system is comprised of multiple inner nuclear membrane proteins, and that Asi2 and Asi3 function in concert with Asi1 to prevent constitutive gene expression. Hence, the Asi proteins have an important regulatory function within the SPS-sensing pathway that is required to maintain the dormant, or repressed state of gene expression in the absence of inducing signals. A model for Asi protein function is schematically presented in Fig. 8.

Our data are consistent with the Asi proteins functioning together in a complex within the inner nuclear membrane. This model is based on several observations. First, mutations in ASI1, ASI2, and ASI3 belong to the same epistasis group. Null mutations in these genes result in identical levels of constitutive SPS sensor-regulated gene expression (Fig. 1A), and identical amino acid uptake-related growth phenotypes (Fig. 2B). Furthermore, all combinations of double and triple mutations give rise to similar levels of gene expression and identical growth phenotypes as the individual single mutations (Figs. 1A and 2B). These observations indicate that the Asi proteins participate in the same regulatory activity, and are functionally interdependent. Second, all three Asi proteins function to restrict the activity of unprocessed Stp1 and Stp2 under non-inducing conditions (Fig. 2). Third, the Asi proteins are integral polytopic membrane proteins (Figs. 3 and 4) that localize to the inner nuclear membrane (Fig. 5). Consistent with our microscopic analysis, all three Asi proteins co-fractionate to membranes of similar density (Fig. 6). Typical of nuclear membrane proteins, the Asi proteins can be solubilized from membranes using the anionic detergent SLS (Fig. 7A), but retain their membrane association in the presence of non-ionic detergents. Fourth,
Asi1 and Asi3 are homologous (Fig. 3) but not redundant proteins (Fig. 2). Finally, Asi1 and Asi3 co-purify (Fig. 7B).

We eliminated several potential mechanisms that could underlie the constitutive expression of SPS-regulated genes in asi mutants. First, the loss of Asi function could facilitate precocious Stp1 or Stp2 processing, however, amino acid-dependent processing of both factors occurs normally in asiA1–3 mutant strains (Fig. 1). Thus, the Asi proteins do not negatively modulate the endoproteolytic activity of the SPS sensor. Second, the Asi proteins could indirectly affect cytoplasmic retention mechanisms. However, similar to our previous findings in asi1 null mutant strains (27), the loss of Asi2 and Asi3 activity did not visibly perturb the intracellular distribution of unprocessed Stp1 or Stp2 (data not shown). Thus, the three Asi proteins do not grossly affect Stp1 and Stp2 localization. Finally, Asi1 and Asi3 possess nucleoplasmically oriented RING motifs at their extreme C termini. RING motifs mediate protein-protein interactions (41), and have been found in several E3 ubiquitin ligases where they induce AAP gene expression after binding promoters (green/white diagonal box) via their DNA binding domains (green boxes). In cells lacking one or all Asi proteins (red cross designates inactivation), the low levels of latent forms of Stp1 and Stp2 that enter the nucleus constitutively induce the expression of SPS-regulated AAP genes. The increased transcription of AAP genes in B and C leads to induced rates of amino uptake.

Our results regarding Asi protein function are entirely consistent with a simple binding mechanism. Accordingly, by sequestering small amounts of full-length Stp1 and Stp2 that reach the nucleus under non-inducing conditions, the Asi proteins would restrict Stp1 and Stp2 from accessing promoters. We have recently shown that the N-terminal regulatory domain of Stp1 associates directly with the Ssy5 endoprotease (23). Using a two-hybrid approach the interaction surface was mapped to the conserved Region II motif of the N-terminal regulatory domain. Our successful use of the two-hybrid approach, coupled to the finding that conserved Region I confers Asi-dependent regulation when fused to a DNA-binding protein (27), encouraged us to evaluate possible interactions between the N-terminal domain of Stp1 and the hydrophilic domains of Asi1, Asi2, or Asi3. However, this approach failed to provide evidence that these proteins interact. This may indeed be the consequence of the Asi proteins functioning together within a multiprotein complex. Stable interactions with Stp1 and Stp2 may depend on contacts between more than one of the Asi proteins.

Recent studies in mammalian cells have revealed that lamin A/C and MAN1 may regulate gene expression independently of chromatin recruitment in a manner that is consistent with our sequestration model (44). Lamin A/C binds c-Fos, an interaction that correlates with decreased AP-1 DNA binding and transcriptional activation (45). Here the negative effect on gene expression is attributed to lamin A/C-mediated c-Fos sequestration, which reduces c-Fos/c-Jun heterodimer formation. The inner nuclear membrane protein MAN1 has been found to antagonize transforming growth factor β and BMP2 signaling by binding Smad transcription factors (46–48). Smads are phosphorylated by activated transforming growth factor β and BMP2 receptors at the plasma membrane. Interestingly, MAN1 preferentially binds hypophosphorylated Smads (48). These
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observations, and the similarity to our findings regarding Asi protein function, raise the possibility that sequestration of transcriptional regulators at the inner nuclear membrane may be a general strategy to negatively modulate gene expression.

Finally, our finding that Asi1, Asi2, and Asi3 localize to the inner nuclear membrane is intriguing from a cell biological perspective. A number of mammalian nuclear membrane proteins have recently been identified (49), and yeast genome-wide approaches have identified numerous non-pore proteins that localize to the inner nuclear membrane (50). In yeast, two membrane proteins that possess nuclear localization sequences have recently been found to pass through nuclear pores in a karyopherin-dependent manner (51). Thus, inner nuclear membrane proteins may be actively transported to the inner membrane. Intriguingly, Asi3 has a single cluster of similar nuclear localization sequence motifs, whereas Asi1 and Asi2 do not. Thus, it will be interesting to examine the significance of the nuclear localization sequence motif in Asi3, and the mechanisms that enable Asi1 and Asi2 to correctly localize. In mammalian cells, it is thought that membrane proteins are retained in the inner membrane via interactions with chromatin and/or nuclear lamins (52, 53). Yeast lack lamins (7), consequently, there must be lamin-independent mechanisms to ensure correct localization and retention of inner nuclear membrane proteins. The advantage of working in a model system devoid of lamins may facilitate future investigations regarding the localization of inner nuclear membrane proteins and provide general insights into lamin-independent mechanisms controlling gene expression. Such findings may have implications for understanding similar processes in other organisms that lack lamins, such as plants.

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