Heat shock protein 70 (Hsp70) is a highly conserved molecular chaperone that assists in the folding of nascent chains and the repair of unfolded proteins through iterative cycles of ATP binding, hydrolysis, and nucleotide exchange tightly coupled to polypeptide binding and release. Cochaperones, including nucleotide exchange factors (NEFs), modulate the rate of ADP/ATP exchange and serve to recruit Hsp70 to distinct processes or locations. Among three nonrelated cytosolic NEFs in *Saccharomyces cerevisiae*, the Bag-1 homolog SNL1 is unique in being tethered to the endoplasmic reticulum (ER) membrane. We demonstrate here a novel physical association between Snl1 and the intact ribosome. This interaction is both independent of and concurrent with binding to Hsp70 and is not dependent on membrane localization. The ribosome binding site is identified as a short lysine-rich motif within the amino terminus of the Snl1 BAG domain distinct from the Hsp70 interaction region. Additionally, we demonstrate a ribosome association with the *Candida albicans* Snl1 homolog and localize this putative NEF to a perinuclear/ER membrane, suggesting functional conservation in fungal BAG domain-containing proteins. We therefore propose that the Snl1 family of NEFs serves a previously unknown role in fungal protein biogenesis based on the coincident recruitment of ribosomes and Hsp70 to the ER membrane.
alleles, linking it to nuclear pore assembly and function (14). Although the role of Snl1 in nuclear transport is unclear, Snl1 may function to recruit and stimulate Hsp70 to support dynamic aspects of the nuclear pore complex. In contrast to the growth, stress sensitivity, and protein-processing defects caused by the loss of Sse1 (Hsp110) or Fes1 (HspBP1) in yeast cells, snl1Δ cells exhibit no detectable phenotypes (20, 25). In this study, we report a novel interaction of Snl1 with the ribosome complex. We present biochemical and genetic evidence that Snl1 binds predominantly to the 60S subunit independently of its interaction with Hsp70. We identify the ribosome binding site within the Snl1 BAG domain as a short lysine-rich motif similar to other known ribosome-associated factors. Finally, we demonstrate within the Snl1 BAG domain as a short lysine-rich motif similar to other known ribosome-associated factors. Finally, we demonstrate the conservation of this interaction among fungal BAG proteins through an analysis of the predicted Snl1 homolog in the pathogenic fungus Candida albicans. We postulate that Snl1 represents an additional method by which fungi recruit Hsp70 to translating ribosomes to support protein biogenesis.

MATERIALS AND METHODS

Cell culture. S. cerevisiae strains were grown in medium containing yeast extract–peptone-dextrose (YPD) at 30°C, unless otherwise indicated. Synthetic complete (SC) medium lacking the appropriate nutrient for plasmid selection was purchased from Sunrise Science Products (San Diego, CA). Standard yeast propagation and transformation procedures were employed (21). Plate growth assays were carried out by serial dilution using 1/10 dilution steps with a starting culture optical density at 600 nm of 1.0, and the culture was transferred with a multipronged replicating tool from a 96-well plate. All strains are described in Table S1 in the supplemental material.

Plasmid construction. SNL1 and SNL1ΔN (Δ40) were amplified from BY4741 genomic DNA by use of standard PCR protocols and cloned into the vector p413TEF by use of 5′ SpeI and 3′ Xhol sites for subsequent cloning into p413TEF. pSnl1ΔN–FLAG was harvested and resuspended in TADM buffer (50 mM Tris [pH 7.5], 50 mM ammonium acetate, 1 mM dithiothreitol [DTT], 5 mM magnesium chloride) with PIs (TADM = 200 mM NaCl) with protease inhibitors (PIs) (2 μg/ml aprotinin, 2 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml chymostatin [Sigma]) (TEGN200 + PI), followed by the addition of acid-washed glass beads. The samples were then lysed by agitation using a microtube mixer for five rounds of 1 min of lysis in each round followed by 1 min on ice. The lysate was then cleared by centrifugation at 3,000 x g at 4°C for 10 min. For KCI treatment, the lysate was split equally into four tubes containing the indicated amounts of KCI (0, 0.1, 0.2, and 0.5 M) and incubated on ice for 30 min.

For immunoprecipitations (IPs), 30 μl of a 1:1 slurry of M2 resin (Sigma) in TEGN200 was added to a lysate prepared from 25 ml of log-phase (Δ600 = 1.0) cultures of the appropriate strains. The volume was adjusted to 700 μl with TEGN200 + PI, followed by incubation with mixing at 4°C for 2 h. Resin was then collected by centrifugation and washed seven times with TEGN + PI. For EDTA treatment, the Rps6B–3′X hemagglutinin (3′XHA) strain (a gift from M. Ptashne, Sloan-Kettering Institute) expressing Snl1ΔN–FLAG was harvested and resuspended in TADM buffer (50 mM Tris [pH 7.5], 50 mM ammonium acetate, 1 mM dithiothreitol [DTT], 5 mM magnesium chloride) with PIs (TADM + PI) and lysed as described above. Snl1ΔN was immunoprecipitated from the cell lysate, and the resin was washed two times with TADM + PI. The FLAG resin was split equally, 40 μl EDTA was added to one tube, and samples were washed by 30 min on ice for 30 min. The FLAG resin was washed two times, and proteins were eluted as mentioned below. For the competition assay, Snl1ΔN–FLAG was immunoprecipitated from strain BY4741 by using anti-FLAG M2 affinity resin. Reombinant His6-Ssb1 (1 mg/ml) was added to Snl1ΔN bound to FLAG resin, and the sample was incubated on ice for 30 min. Another IP was done for 1 h, and bound proteins were eluted as mentioned below.

For the elution of bound proteins, 35 μl of 200 μg/ml FLAG peptide (GenScript) in TEGN200 was added to the resin pellet and incubated at room temperature for 15 min with occasional shaking. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained or Coomassie blue dye or transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) for immunodetection. Anti-Ssa1/2 polyclonal antibody was used at a 1:40,000 dilution (a gift from M. Ptashne, Sloan-Kettering Institute). Anti-Ssb1/2 polyclonal antibody was used at a 1:5,000 dilution (a gift from E. Craig, University of Wisconsin). M2 monoclonal antibody (which recognizes the FLAG epitope; Sigma) was used at a 1:1,000 dilution. Anti-Rpl8 antibody was used at a 1:2,000 dilution (a gift from A. Johnson, University of Texas, Austin), and anti-Rpl3 antibody was used at a 1:1,000 dilution (a gift from J. Warner, Albert Einstein College of Medicine). Rp56B-3′XHA was detected by using an anti-HA antibody at a 1:2,000 dilution.

Microscopy. Candida albicans cells were harvested by centrifugation, washed once with water, and resuspended in 70% ethanol for fixation for 1 min. Cells were once again harvested by centrifugation and resuspended in water for visualization. Nuclei of living cells were stained with the vital DNA dye Hoechest 33342 at a concentration of 10 μg/ml for 5 min. Cells were observed under a BX60 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a 100X immersion oil objective and 4′,6-diamidino-2-phenylindole or GFP filter cubes. Images were captured with a Photometrics CoolSNAP-fx cooled charge-coupled-device camera driven by QED image-capturing software (Media Cybernetics, Bethesda, MD). All images were processed by using Photoshop CS (Adobe Systems, San Jose, CA).
Protein identification. Proteins were identified at the Proteomics Core Facility at the University of Texas Health Science Center at Houston. Tandem mass spectrometry (MS/MS) analysis was performed with an Applied Biosystems QStar Elite liquid chromatography-MS/MS (LC/MS/MS) mass spectrometer equipped with an LC Packings high-performance liquid chromatography (HPLC) instrument for capillary chromatography. The HPLC instrument was coupled to the mass spectrometer by a Nanospray II electrospray ionization (ESI) source for direct analysis of the eluate. For protein identification, bands of interest were excised from a Coomassie-stained gel, cysteines were blocked with iodoacetamide, and the gel pieces were dried after washing with acetonitrile. Proteolytic digestion was carried out overnight with trypsin (12.5 ng/ml in 25 mM NH₄H₂CO₃) at 37°C. After digestion, peptides were extracted with 30 μl 50% acetonitrile–5% formic acid, dried, and redissolved in 5 μl 2% acetonitrile–0.1% formic acid with vortexing. Peptides were separated by using an UltiMate nano-HPLC instrument and a Famos autosampler (Dionex LC Packings) and identified by nano-LC/MS/MS on a QSTAR Elite mass spectrometer (AB Sciex Instruments) operating in the positive-ion mode. Peptides were identified by Protein Pilot (Applied Biosystems) and verified with MASCOT with MS and MS/MS mass tolerance of 50 ppm and 0.1 Da, respectively.

RESULTS
Snl1 is associated with intact ribosomes. It was previously shown that the cytosolic BAG domain homology region of Snl1 interacts in vitro with the yeast Hsp70s Ssa and Ssb (31). This interaction is consistent with the canonical BAG domain-containing protein Bag-1 in mammals, which interacts directly with the N-terminal ATPase domain of Hsc70 (36, 44). However, whether Snl1 associates with the cytosolic Hsp70s in vivo was unknown. To test this, we constructed expression vectors to produce FLAG-tagged full-length Snl1 and a truncated variant of Snl1 (Snl1ΔN) that lacks the transmembrane domain but contains the entire cytosolic region, including the conserved BAG domain. We then affinity purified both proteins using M2-agarose resin from whole-cell extracts solubilized with 1% Triton X-100 to release membrane-associated Snl1. We have previously demonstrated that this affinity purification method works efficiently in yeast to determine chaperone interactions in vivo (29). In addition to the Snl1 proteins, we independently expressed FLAG-tagged Sse1, Sse2, and Fes1 fusions from the identical promoter to compare Hsp70 copurification patterns. As shown in Fig. 1A, at least 20 additional bands copurified with Snl1 and Snl1ΔN in addition to Ssa and Ssb. In contrast, these proteins were absent when FLAG-Sse1, FLAG-Sse2, or FLAG-Fes1 was isolated, while Ssa and Ssb were identified in all the pulldowns. We also noted that the amount of Ssa was disproportionately reduced in the two Snl1 purifications relative to the amounts of the other three NEFs. In addition, FLAG-Fes1 appeared to associate specifically with Ssa in vivo, with little to no Ssb copurifying. We utilized mass spectrometry to identify the individual Snl1-copurifying bands and discovered that they all could be assigned as large (L) and small (S) subunits of the ribosome that were identified by using mass spectrometry. EV, empty vector.

FIG 1 Snl1 interacts with ribosomal proteins. (A) Amino- or carboxyl-terminally FLAG (F)-tagged NEFs were immunoprecipitated from wild-type (BY4741) whole-cell lysates as described in Materials and Methods. Immunoblot analysis was used to detect proteins interacting with anti-Ssa, anti-Ssb, and anti-Rpl8 antisera. Red letters represent proteins of the large (L) and small (S) subunits of the ribosome that were identified by using mass spectrometry. EV, empty vector. (B) The whole-cell lysate from BY4741 expressing Snl1ΔN-FLAG was split equally and incubated on ice for 15 min with the indicated concentrations of KCl. Snl1ΔN was immunoprecipitated, and interacting proteins were separated on a 15% SDS-PAGE gel. Immunoblot analysis was used to detect Rpl3 and Snl1ΔN (anti-FLAG antibody). CBB, Coomassie brilliant blue; IP, FLAG immunoprecipitation; IB, immunoblot of the whole-cell lysate.
against the large-subunit protein Rpl8. This experiment verified a robust interaction between Snl1 (membrane-associated and soluble forms) and the ribosome and failed to detect any interaction with the other NEFs that may have been below the Coomassie detection threshold. These results reveal a novel interaction between a membrane-associated Hsp70 NEF in *S. cerevisiae* and the assembled 80S ribosome. These results also indicate that ribosome binding by Snl1 is independent of its attachment to the ER membrane.

The ribosome-associated Hsp70 Ssb has been shown to maintain an association with ribosomes after a high-salt wash procedure (1 M KCl) frequently used to remove loosely affiliated proteins (28). We therefore sought to determine whether ribosome copurification with Snl1 was dependent on Ssb. Because the two Ssb proteins are not essential, we first determined whether the Snl1ΔN-ribosome interactions occurred in a strain lacking these Hsp70 genes. Snl1ΔN-FLAG was expressed and isolated from wild-type and ssb1Δ/Δ ssb2Δ/Δ strains, as shown in Fig. 2A. We found that Snl1 remained associated with the ribosome in the ssb1Δ/Δ ssb2Δ/Δ strain, as verified by immunoblotting for Rpl3. With a parallel approach, we took advantage of mutations shown previously to abrogate binding to Ssa and Ssb by constructing a FLAG-tagged allele of SNL1 containing alanine substitutions at two residues critical for Hsp70 binding (Snl1ΔN-FE112A,R141A) (31). To assess if Snl1ΔN interacted with the ribosome in the absence of Hsp70 binding, we immunoprecipitated the mutant variant and the isogenic wild type as a control. In corroboration with previous findings, we found that this mutant exhibited considerably reduced binding to both Ssa and Ssb in vivo, as shown by the respective immunoblots (Fig. 2B). In contrast, binding to ribosomes, as assessed by anti-Rpl3 immunoblotting, was unaffected. Taken together, these two independent approaches demonstrate that Hsp70 does not mediate the association of Snl1ΔN with the ribosome.

**Interaction of Snl1 with the ribosome is Hsp70 independent.** Because Snl1 associates with the ribosome-associated Hsp70 Ssb, we sought to determine whether ribosome copurification with Snl1 was dependent on Ssb. Because the two Ssb proteins are not essential, we first determined whether the Snl1ΔN-ribosome interactions occurred in a strain lacking these Hsp70 genes. Snl1ΔN-FLAG was expressed and isolated from wild-type and ssb1Δ/Δ ssb2Δ/Δ strains, as shown in Fig. 2A. We found that Snl1 remained associated with the ribosome in the ssb1Δ ssb2Δ strain, as verified by immunoblotting for Rpl3. With a parallel approach, we took advantage of mutations shown previously to abrogate binding to Ssa and Ssb by constructing a FLAG-tagged allele of SNL1 containing alanine substitutions at two residues critical for Hsp70 binding (Snl1ΔN-FE112A,R141A) (31). To assess if Snl1ΔN interacted with the ribosome in the absence of Hsp70 binding, we immunoprecipitated the mutant variant and the isogenic wild type as a control. In corroboration with previous findings, we found that this mutant exhibited considerably reduced binding to both Ssa and Ssb in vivo, as shown by the respective immunoblots (Fig. 2B). In contrast, binding to ribosomes, as assessed by anti-Rpl3 immunoblotting, was unaffected. Taken together, these two independent approaches demonstrate that Hsp70 does not mediate the association of Snl1ΔN with the ribosome.

**The ribosome and Hsp70 exhibit noncompetitive binding to Snl1.** The above-described results demonstrate that the Snl1 asso-
cation with ribosomes does not require Hsp70 but do not establish whether this binding occurs on a novel or shared binding interface. To investigate this question, we performed a binding competition experiment using purified Hsp70. Snl1ΔN-ribosome-Hsp70 complexes were isolated by using M2 resin and mixed with excess recombinant His6-Ssb or buffer alone and then washed and resolated prior to recovery by elution. As shown in Fig. 2C, Snl1ΔN immunoprecipitated from cell extracts copurified with endogenous Ssb and the ribosome. When exogenous His6-Ssb was added, it outcompeted the binding of endogenous Ssb to Snl1ΔN as expected, as assessed by the slower migration likely caused by the His6 tag (α-Ssb panel, compare top and bottom bands). However, the interaction with the ribosome was unaffected. These findings suggest that Hsp70 and the ribosome interact with Snl1 in a noncompetitive manner, consistent with the presence of two distinct and independent binding sites.

**Snl1 binds to the 60S ribosomal subunit.** Our initial mass spectrometric analysis demonstrated the presence of protein components of both the large and small ribosomal subunits copurifying with Snl1. To distinguish which subunit might be interacting directly with Snl1, we treated the copurified protein complexes with EDTA. EDTA treatment is a well-characterized method of separating the two subunits by virtue of the chelation of Mg2+ ions required for 60S-40S subunit assembly (2). Snl1ΔN-FLAG was expressed in and immunoprecipitated from a yeast strain containing a chromosomally epitope-tagged ribosomal small subunit (Rps6B-3×HA) to allow the detection of the 40S component. Protein complexes were incubated in the absence or presence of 40 mM EDTA and washed, and bound proteins were eluted. We noted a dramatic decrease in the amount of Rps6B-3×HA coeluting with Snl1ΔN-FLAG, with very little change in the signal for Rpl8. We interpret these results to indicate that Snl1 predominantly binds to the large (60S) ribosomal subunit.

**Identification of the ribosome binding site in Snl1.** The above-described findings demonstrated that the ribosome binding site within Snl1 was distinct from its Hsp70 binding site. The BAG domain is composed of a three-helix bundle, with the majority of Hsp70 contacts being localized to helices 2 and 3 (32). Because our data suggest that Snl1 can concomitantly bind both Hsp70 and the ribosome, steric hindrance would likely preclude both binding sites being located within these two helices. To identify the ribosome binding site, we therefore constructed a series of amino-terminal truncation mutants by sequentially deleting 10 residues starting at the beginning of putative helix 1 of Snl1 (Fig. 3A). All constructs were individually expressed in yeast cells and FLAG immunoprecipitated. With the exception of the Δ80 mutant, all the truncation proteins appeared to be stably expressed (Fig. 3B). In addition, all but the Δ80 mutant retained binding to Ssa and Ssb, as judged by Coomassie staining, suggesting that they were properly folded. Strikingly, we noted that the ribosome interaction was lost in the Δ60 and Δ70 truncation mutants, as indicated by Coomassie staining and anti-RpL3 immunoblotting. These results suggested that residues required for ribosome binding were found between residues 50 and 60. An evaluation of the amino acids in this region revealed a concentration of five lysine residues (residues 52 to 58), which would be expected to provide a positively charged binding interface, not uncommon in ribosome binding proteins (41, 43) (Fig. 3A). To examine directly if this region was responsible for ribosome binding, we constructed a mutant of Snl1ΔN in which the five lysine residues were replaced with alanine (5KA) (Fig. 3A). We immunoprecipitated Snl1ΔN-5KA and Snl1ΔN from yeast cell extracts. As assessed by Coomassie staining and immunoblotting, the 5KA mutant exhibited drastically reduced ribosome associations. In contrast, interactions with Ssa and Ssb remained unchanged. These results demonstrate that this positively charged region within Snl1 is specifically required for ribosome interactions but not Hsp70 binding.

**Conservation of ribosome associations in a fungal BAG-1 homolog.** The identification of a specific sequence within Snl1 required for ribosome binding prompted us to ask whether this was a conserved or unique feature of Bag-1 homologs. As shown in Fig. S1 in the supplemental material, putative Bag-1 homologs could be identified in a range of eukaryotic organisms, many of which possess positively charged regions in the amino terminus of the predicted BAG domain. The genome of the human-pathogenic fungus *C. albicans* encodes a single predicted protein with homology to the BAG domain, (orf19.997). This protein bears a high level of sequence conservation (32% identity and 59% similarity) with *S. cerevisiae* SNL1, including a putative N-terminal transmembrane region and a lysine-rich region in putative helix 1 of the BAG domain (Fig. 4A). This is in contrast to the mammalian Bag-1 protein (short isoform), which, despite significant sequence homology (25% identity), lacks both features. Together, these analyses led us to speculate that the *C. albicans* Snl1 (CaSnl1) homolog (i) would localize to the ER and (ii) would associate with ribosomes. We tested these predictions by first carboxy-terminally tagging one copy of the gene in *C. albicans* with GFP using a method described previously (10). Due to the dearth of available fluorescent organellar localization markers in this organism, we stained live cell nuclei with the DNA-intercalating Hoechst dye to identify the nucleus. Untagged control cells exhibited discrete nuclear staining, with no background signal in the green channel. In contrast, CaSnl1-GFP was clearly seen to localize around the nucleus and at the cell periphery, entirely consistent with known perinuclear and cortical ER localization patterns and with previous reports of the localization of Snl1 in *S. cerevisiae* (Fig. 4B) (14, 16).

Because the lysine-rich region in *S. cerevisiae* Snl1 was shown to be required for ribosome binding, we reasoned that the similar sequence within CaSnl1 would confer ribosome interactions. To test this prediction, we generated two truncated forms of the Snl1 homolog from *C. albicans* strain SC5314 and expressed them in *S. cerevisiae* cells. CaSnl1Δ91 lacks the first 90 amino acids, including the transmembrane domain, but contains the lysine-rich region, whereas CaSnl1Δ103 lacks both domains (Fig. 4A). A murine Bag-1 cDNA was likewise expressed in yeast to ascertain whether the absence of a lysine-rich region in a distinct Bag-1 homolog would preclude ribosome interactions. We observed that the *C. albicans* Snl1 homolog exhibited the same characteristics for ribosome binding as *S. cerevisiae* Snl1, with both variants binding Hsp70 but only the Δ91 construct binding ribosomes (Fig. 4C). In addition, murine Bag-1 likewise associated with Hsp70, demonstrating the high level of conservation within the Hsp70 binding domain, but failed to interact with yeast ribosomes. These experiments demonstrate that a lysine-rich region within putative helix 1 of the BAG domain mediates interactions with assembled 80S ribosomes in two distantly related fungi, whereas this interaction is not conserved in the dominant Bag-1 isoform in mammalian cells.
We report here for the first time an interaction between the *S. cerevisiae* BAG domain protein Snl1 and the ribosome. In addition, we show that this novel interaction is conserved in the *C. albicans* Snl1/Bag-1 homolog, implying a conserved function. Snl1 is unique in being the only known membrane-anchored Hsp70 nucleotide exchange factor, and we found that ribosomes tightly associate with both the membrane-associated and soluble truncated Snl1/H9004N isoforms. We therefore conclude that the attachment of Snl1 to the ER/nuclear membrane does not occlude the ribosome binding site and, as a corollary, that the localization of Snl1 at the membrane does not dictate ribosome binding. Using the same affinity purification approach, we determined that Snl1 was unique among the *S. cerevisiae* cytosolic NEFs in ribosome associations, as we did not detect binding with the Sse1, Sse2, or Fes1 protein. In contrast, all four factors readily associated with Hsp70, verifying that the addition of the FLAG affinity tag does not noticeably distort protein-protein interactions.

**Fungi are unique in having a dedicated ribosome-associated Hsp70 (Ssb), in contrast to other cell types, which recruit a general cytosolic Hsp70 to assist in nascent-chain folding via an intermediate tether protein. However, our results suggest that the interaction of Snl1 with the ribosome is independent of its association with Hsp70 and its function as a NEF. First, we found that Snl1 binding to Hsp70 was salt resistant, whereas the binding to ribosomes was largely abolished at high salt concentrations (0.5 M KCl). Second, the genetic elimination of both Ssb1 and Ssb2 had no effect on the Snl1-ribosome interaction, and the addition of exogenous Ssb to purified Snl1-ribosome-Ssb complexes likewise did not reduce the Snl1-ribosome association. Third, we observed**

![Diagram](https://example.com/diagram.png)

**FIG 3** Snl1 interacts with the ribosome via a lysine-rich-region binding motif. (A) Truncations within putative helix 1 of the Snl1 Bag domain are depicted. TM, putative transmembrane domain. The lysine-rich region between residues 50 and 60 in Snl1 is highlighted, and the residue changes that result in the 5KA mutant are indicated. (B) FLAG (F)-tagged Snl1ΔN truncations were immunoprecipitated from the wild-type (BY4741) whole-cell lysate. Immunoblot analysis was used to detect ribosomal proteins with anti-Rpl3 antisera. (C) FLAG-tagged Snl1ΔN, either the wild type (WT) or the 5KA mutant, was immunoprecipitated from BY4741 cells, and immunoblot analysis was used to detect stably produced proteins with anti-Rpl3 antisera. IP, FLAG immunoprecipitation; IB, immunoblot of the whole-cell lysate.
that a previously characterized mutant of SNL1 with amino acid substitutions shown to abolish Hsp70 interactions (E112A R141A) retained the ribosome association. Fourth, we identified the ribosome binding interface in Snl1 and localized it to residues 52 to 58 within the predicted helix 1 based on homology to mammalian Bag-1, distinct from the Hsp70-interacting residues located in helices 2 and 3. These interactions invite comparisons to the recruitment of Ssb by the ribosome-associated complex (RAC) composed of Ssz1 and Zuo1 in fungi and the recently demonstrated recruitment of cytosolic Hsp70 in mammals by the Zuo1 ortholog Mpp11 (Fig. 5) (17, 43). In both cases, these ribosome-associated proteins serve two roles: (i) tethering an Hsp70 chaperone to the ribosome and (ii) stimulating Hsp70 cycling via the acceleration of its intrinsic ATPase activity. Ribosome-associated Snl1 would be predicted to fulfill both these roles, stimulating

FIG 4 Ribosome association is conserved in the sole BAG domain-containing protein of Candida albicans. (A) Illustration depicting the sequence alignment of BAG domain-containing proteins of Saccharomyces cerevisiae and Candida albicans showing the lysine-rich region at the N terminus and the predicted transmembrane (TM) domains. Murine Bag-1 lacks both a transmembrane domain and a Lys-rich region. Mm, Mus musculus. (B) Localization of the chromosomally tagged C. albicans Snl1 homolog (CaSnl1-GFP) in wild-type C. albicans strain SC5314 was determined by fluorescence microscopy. Cells were stained with Hoechst 33342 dye, as described in Materials and Methods, to facilitate the assessment of perinuclear ER localization. (C) Snl1ΔN-FLAG from S. cerevisiae and the corresponding truncations in the C. albicans Snl1 homolog and the mammalian Bag-1 protein were expressed and immunoprecipitated from the wild-type (BY4741) yeast whole-cell lysate. Proteins were separated on a 15% SDS-PAGE gel, and immunoblot analysis was used to detect ribosomal proteins with anti-Rpl8 antisera. CBB, Coomassie brilliant blue; IP, FLAG immunoprecipitation; IB, immunoblot of the whole-cell lysate.

FIG 5 Hsp70 recruitment by ribosome-associated cofactors. Hsp70 protein chaperones are shown to be recruited to intact, translating ribosomes via interactions with ribosome-associated factors. Mpp11 and RAC (Zuo1) are J proteins, whereas Snl1 is a nucleotide exchange factor. Both proteins bind Hsp70 within the NBD and accelerate its intrinsic ATPase activity.
Hsp70 ATPase activity via its role as a potent nucleotide exchange factor. Genes encoding ribosomal or ribosome-associated proteins are frequently coregulated at the transcriptional level (23). Using the Serial Pattern of Expression Levels Locator (SPELL) database, we found that SNL1 transcript levels correlated well with those of ribosomal protein genes when queried over all available data sets (20 of the top 50 gene hits) (see Table S3 in the supplemental material) (13). These transcriptional data support our contention that Snl1 is a bona fide ribosome-associated factor and suggest that cells may regulate levels of Snl1 to match protein biosynthetic needs.

Results from both mass spectrometry and immunoblot analyses indicate that Snl1 interacts with assembled ribosomes, as we detected associations with both large (60S)- and small (40S)-subunit proteins. The treatment of affinity-purified Snl1-ribosome complexes with the divalent cation chelator EDTA resulted in the release of the small subunit, while binding to the large subunit was maintained. We therefore favor a model wherein Snl1 associates directly with one or more large-subunit components via the lysine-rich region that we have identified. A surface-exposed motif composed of positively charged residues [RRK(X)KK] mediates the ribosome association by the β-subunits of the nascent-chain-associated complex (NAC) in yeast and mammals (41, 42). A similar charged interface is utilized for ribosome associations by the bacterial trigger factor (TF) (22). Our identification of the lysine-rich region required for ribosome binding by Snl1 is thus consistent with previously described ribosome-associated chaperone systems. Based on homology to mammalian Bag-1, this region is predicted to form helix 1 of a three-helix bundle. In contrast, the ribosome binding sites within the NAC and TF lie within flexible loop domains. Structure determination of the Snl1 cytosolic domain will shed light on the precise geometry of the Snl1-ribosome interaction, which is of particular importance given the proximity of the Hsp70 binding domain within the BAG motif. In addition, sequence heterogeneity within the amino terminus of BAG domain-containing proteins may contribute to their functional diversification (37). The stress-signaling kinase Raf-1 binds Bag-1 directly, and the binding sites for Raf-1 and Hsc70 on Bag-1 overlap, dictating that the two proteins interact with Bag-1 in a mutually exclusive manner (33). Thus, the ability of a BAG domain to mediate binding to multiple partners is not without precedent.

How widespread is the interaction between BAG domain proteins and the ribosome? In this report, we demonstrate that the Snl1/Bag-1 homolog in the pathogenic fungus Candida albicans is capable of binding the intact ribosome when expressed in S. cerevisiae. Moreover, we verify that this homolog is membrane associated in C. albicans via fluorescence microscopy utilizing a CaSnl1-GFP fusion. Interestingly, the genome of the fission yeast Schizosaccharomyces pombe contains two predicted Bag domain-containing proteins, only one of which possesses a positively charged region. The human Bag-1 protein is expressed as different isoforms that arise from a single mRNA by alternative translation initiation (1, 27). The Bag-1 isoform used in our experiments was the 207-residue short isoform, Bag-1S. Sequence analysis of the medium and large isoforms, denoted Bag-1M and Bag-1L, respectively, revealed a short, positively charged sequence at their N termini that was absent from Bag-1S (see Fig. S1 in the supplemental material). It is therefore possible that cells may express distinct Bag-1 proteins with or without the capacity to associate with ribosomes.

What roles might Snl1 be playing as a ribosomal membrane tether? Given its location at the ER membrane, a role in the transport of proteins from the cytoplasm into the ER membrane or lumen can be envisioned. Because cotranslationally translocated proteins are recruited to the ER and the translocon machinery via the signal recognition particle (SRP) system, it seems unlikely that Snl1 would contribute to this pathway. In contrast, posttranslationally translocated proteins are released into the cytoplasm after synthesis and must be directed to the translocon for import. At least one posttranslationally translocated protein in S. cerevisiae, pre-pro-α-factor (ppαF), also requires cytosolic Hsp70 (Ssa1) to be maintained in a translocation-competent state (4). Therefore, the concurrent ER targeting of both ribosomes and Hsp70 via Snl1 would likely enhance translocation by increasing the local concentrations of both factors. In an analogous scenario, the mitochondrial inner membrane protein Oxa1 is required for the proper insertion of respiratory-chain complexes synthesized on matrix-localized ribosomes and interacts with these ribosomes via a positively charged carboxyl-terminal motif (19, 35). Thus, Oxa1 could likewise promote substrate protein maturation in a nonspecific manner via ribosome recruitment. The formal testing of such hypotheses is confounded by the lack of detectable functional phenotypes in cells lacking SNL1. For example, a preliminary analysis of ppαF processing failed to demonstrate a major defect in snl1Δ cells (data not shown). In addition, we were unable to detect synthetic genetic phenotypes when SNL1 was deleted in combination with RAC, NAC, SSB, or ER translocation mutants (see Fig. S2 and S3 in the supplemental material). Additionally, we were unable to recapitulate the synthetic phenotypes reported by a recently published high-throughput whole-genome screen (see Fig. S4 in the supplemental material) (3). It is therefore likely that the yeast Bag-1 homolog plays a subtle or condition-specific role in protein biogenesis that is nevertheless important enough to be conserved across more than 250 million years of separation between S. cerevisiae and C. albicans. Revealing such a role may require unbiased genomewide and/or proteome-wide studies, as was recently done for the role of the NAC, which remained enigmatic for a number of years after its discovery and is still not completely resolved (7). Snl1 has been linked to nuclear pore function through its identification as a high-copy-number suppressor; it is therefore possible that it may also be involved in ribosome biogenesis and export, a function recently attributed to Ssb and NAC proteins. Despite the intriguing discovery of the novel interaction between fungal Bag proteins and the ribosome, it is clear that significant additional work is required to understand the importance of this association. Nevertheless, our discovery of Snl1 as a possible bridge between translating ribosomes and Hsp70 represents an important step in an understanding of the biology of this family of proteins.

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