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## The *Candida albicans* Kar2 protein is essential and functions during the translocation of proteins into the endoplasmic reticulum

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### Abstract

Since the secretory pathway is essential for *Candida albicans* to transition from a commensal organism to a pathogen, an understanding of how this pathway functions may be beneficial for identifying novel drug targets to prevent candidiasis. We have cloned the *C. albicans* *KAR2* gene, which performs many roles during the translocation of proteins into the endoplasmic reticulum (ER) during the first committed step of the secretory pathway in many eukaryotes. Our results show that *C. albicans* *KAR2* is essential, and that the encoded protein rescues a temperature-sensitive growth defect found in a *Saccharomyces cerevisiae* strain harboring a mutant form of the Kar2 protein. Additionally, *S. cerevisiae* containing *CaKAR2* as the sole copy of this essential gene are viable, and ER microsomes prepared from this strain exhibit wild-type levels of post-translational translocation during in vitro translocation assays. Finally, ER microsomes isolated from a *C. albicans* strain expressing reduced amounts of *KAR2* mRNA are defective for in vitro translocation of a secreted substrate protein, establishing a new method to study ER translocation in this organism. Together, these results suggest that *C. albicans* Kar2p functions during the translocation of proteins into the ER during the first step of the secretory pathway.

### Keywords

*KAR2*; Secretory pathway; *Candida albicans*; ER translocation; Translocation assay

### Introduction

The pathogenic yeast *Candida albicans* causes many forms of human disease, especially in immunocompromised individuals (Ruhnke 2002; Kullberg and Filler 2002; Filler and Kullberg 2002). In order for this organism to switch from its avirulent commensal state to a virulent one, a number of physiological changes must occur. These changes include the morphogenesis of at least a subset of cells from a budding yeast form to pseudohyphal and true hyphal morphologies (Sudbery et al. 2004; Romani et al. 2003). This transition requires the secretion of new cell wall proteins that are important for cell wall expansion (Chaffin 2008; Chaffin et al. 1998; Rico et al. 1991), as well as for aiding in adhesion to host tissues (Tronchin et al. 1991) and implanted medical devices (Meda et al. 2007; Verstrepen and Klis 2006). In addition to secreted cell wall proteins, infectious forms of *C. albicans* also

require the secretion of aspartyl proteases (reviewed in Gagnon-Arsenault et al. 2006; Sohn et al. 2006; Naglik et al. 2003; Hube 1996) and various lipases and phospho-lipases (reviewed in Schaller et al. 2005) that presumably aid in host tissue invasion. Thus, protein secretion is a key component of the virulence arsenal of *C. albicans*.

In support of the virulence role for protein secretion, many specific *C. albicans* proteins that function in the secretory pathway have been shown to be important for the transport of virulence factors and the establishment of virulence characteristics in this fungus. For example, Vps1p (Bernardo et al. 2008) and Vps4p (Lee et al. 2009; Thomas et al. 2009) are *C. albicans* vacuolar protein sorting proteins that have been observed to be involved in protease secretion, filamentation, biofilm formation and in vivo virulence. Additionally, the deletion of the *C. albicans* *PMR1* gene, which is involved in Golgi-level glycosylation of secretory proteins, leads to a decrease in virulence in a murine system (Bates et al. 2005). Finally, secreted *C. albicans* proteins have been found to affect the morphology and motility of infected cultured epithelial cells (Sandovsky-Losica and Segal 2006). Because protein secretion is essential for the establishment of *C. albicans* infections, an understanding of the mechanisms of protein secretion in this organism could result in the identification of new chemotherapeutic targets.

Despite the fact that the transition from the a virulent form of *C. albicans* to a pathogenic one requires the secretion of proteins to the cell surface and surrounding matrix, few studies have investigated the basic biology of the early secretory pathway in *C. albicans*, namely the translocation of secretory proteins into the endoplasmic reticulum (ER; reviewed in Fonzi 2009). A few *C. albicans* proteins presumed to play roles in the early secretory pathway have been shown to function similarly to their *Saccharomyces cerevisiae* orthologs, such as *SEC65* (Regnacq et al. 1998) and *SPC3*, an ER resident protein involved in N-terminal signal sequence cleavage (De la Rosa et al. 2004a). Other *C. albicans* proteins that are involved later in the secretory pathway have also been shown to function similarly to their *S. cerevisiae* homologues, including *SEC4* (Mao et al. 1999; Clément et al. 1998), *SEC14* (Monteoliva et al. 1996), *SEC18* (Nieto et al. 1993) and *VPS1* (Bernardo et al. 2008). Interestingly, a few *C. albicans* proteins involved in the secretory pathway do not possess the ability to complement the function of their *S. cerevisiae* homologues. Genes encoding such proteins include *SEC61* (De la Rosa et al. 2004b) and *SEC20* (Weber et al. 2001). While there may be multiple explanations as to why these *C. albicans* proteins do not complement their *S. cerevisiae* homologues, these results suggest that one cannot know beforehand whether a given gene will or will not function similarly to its *S. cerevisiae* homologue. Given the importance of *C. albicans* as a human pathogen, it seems pertinent to study the mechanisms of protein secretion in *C. albicans*.

The first committed step in the secretory pathway is the translocation of presecretory proteins into the ER, and in *S. cerevisiae* a key player in this process is the Kar2 protein. This ER luminal Hsp70 molecular chaperone protein (Normington et al. 1989; Rose et al. 1989) is involved in many aspects of both post- and co-translational translocation (Matlack et al. 1999; Gething 1999; Brodsky et al. 1995), as well as during the retrotranslocation of aberrant soluble proteins out of the ER and back to the cytosol for degradation (Nishikawa et al. 2005; 2001; Kabani et al. 2003; Plemper et al. 1997), a process which appears to involve many of the same proteins in *C. albicans* (Wimalasena et al. 2008). During import processes in *S. cerevisiae*, the Kar2 protein has been shown to aid in the translocation of presecretory proteins into the ER lumen through the Sec61p complex translocation pore (Matlack et al. 1999; Brodsky et al. 1995; Panzner et al. 1995; Brodsky and Schekman 1993; Sanders et al. 1992), to serve as a gate that “plugs” the translocon from the luminal side of the ER membrane (Alder et al. 2005; Haigh and Johnson 2002; Hamman et al. 1998), and to act as a

chaperone that aids in the folding of presecretory proteins once they are fully translocated into the ER lumen (Gething 1999; Simons et al. 1995).

To date, the *KAR2* gene in *C. albicans* has not been directly characterized. Because of the many roles that Kar2p plays in the early secretory pathway in other eukaryotes, and because the secretory pathway is critical for *C. albicans* virulence, we have cloned the *KAR2* gene from *C. albicans* and begun to characterize the function of the encoded protein. In this study, we have identified and initially characterized orf19.2013 in the *C. albicans* genome that encodes a predicted protein that is 71% identical and 81% similar to *S. cerevisiae* Kar2p at the amino acid level. Our results indicate that *C. albicans* Kar2p is essential, much like its *S. cerevisiae* ortholog. In addition, *C. albicans* Kar2p functions similarly to its *S. cerevisiae* ortholog, and *C. albicans* microsomes containing reduced amounts of Kar2p are defective for the translocation of a secretory protein substrate. Together, these results indicate that *C. albicans* Kar2p functions during the translocation of secretory proteins into the ER of this human pathogen and is likely critical for this fungus to cause disease in humans.

## Materials and methods

### Strains and general methods

Strains used in these studies are listed in Table 1. Routine DNA and protein methods used in this study were performed following standard protocols generally as outlined in Sambrook and Russell (2001).

### Cloning of the *C. albicans* *KAR2* gene

Genomic DNA was isolated from *C. albicans* strain NIH3172 (ATCC, Manassas, VA; ATCC #14053). This strain was grown in 7.0 mL of YPD (1% yeast extract, 2% bacto-peptone, 2% dextrose) overnight at 30°C. The cells were then harvested, washed with water, sedimented as before and then resuspended in 200  $\mu$ L of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA), 200  $\mu$ L of phenol/chloroform and 300  $\mu$ L of 425–600  $\mu$ m glass beads (Sigma–Aldrich, St. Louis, MO). The tubes were then agitated in a vortex mixer for 4 min followed by the addition of 200  $\mu$ L of TE (10 mM Tris pH 7.6, 1 mM EDTA) and centrifugation in a microcentrifuge for 5 min. The aqueous layer was then removed and the DNA was ethanol precipitated. The pellet was suspended in TE, treated with RNase A and ethanol precipitated again. The resulting DNA pellet was then suspended in 50  $\mu$ L of TE and stored at –20°C.

In order to clone the *C. albicans* *KAR2* gene, which lacks CUG codons in the encoded mRNA, a standard PCR approach was employed. Genomic DNA from *C. albicans* strain NIH3172 was used in PCR reactions containing primers fcaKAR2bam (5'-GCATGGATCCCAACTTACCTTCCACGGTTTT-3') and rcaKAR2sal (5'-GCATGTCGACGGTCCGTTTCATTTTCATGGCT-3') (restriction sites are underlined; primers designed using the Candida Genome Database (CGD), <http://www.candidagenome.org/>), which amplified a 2,414 bp fragment containing the *CaKAR2* gene flanked by *Bam*HI and *Sal*I restriction sites. The PCR product was purified using a PCR purification kit (Qiagen, Valencia, CA). Next, the PCR product and the yeast expression vector pYEX-BX (Clontech, Mountain View, CA) were cut with *Bam*HI and *Sal*I (Fermentas, Hanover, MD) in double digests followed by a purification reaction using the PCR purification kit. Ligation reactions were then performed overnight at 23°C. Ligation products were transformed into competent DH5 $\alpha$  cells and plated on LB + 50 mg/mL ampicillin. Plasmid minipreps were performed and the correct ligation product was confirmed using a diagnostic *Pst*I digestion. The resulting plasmid was named pCaKar2-BX.

### Construction of a conditional *kar2* null mutant in *C. albicans*

The *C. albicans* BWP17 strain was routinely cultured on YPD. The selective medium was YNB (1.2% Difco yeast nitrogen base, 5% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10% succinic acid, 6% NaOH, 2% glucose) supplemented with the appropriate amino acids and/or uridine at a concentration of 80 µg/ml. All strains were grown at 30°C.

Plasmid pCaDIS-KAR2 was constructed as follows. A 1,000 bp fragment of the 5' end of the *C. albicans* *KAR2* gene was PCR amplified from BWP17 genomic DNA, using Deep Vent Polymerase (New England Biolabs, Ipswich, MA) and primers F-CA-KAR2-BamHI (5'-GCATGGATCCATGAGATCATTACAATCTTCTT-3') and R1 kb-CA-KAR2-BamHI (5'-GATAGGATCCCATCAACAAAGGAGTCGA-3') (restriction sites are underlined, primers designed using CGD). The PCR product was digested with *Bam*HI, purified with a Qiagen PCR cleanup kit, and ligated into pCaDIS (Care et al. 1999). pCaDIS-KAR2 was then linearized with *Kpn*I and transformed into strain BWP17 (Wilson et al. 1999) using the lithium acetate/PEG protocol outlined by Gietz and Woods (2002).

Clone CAGA643 was obtained as part of the TIGR library of transposon insertions into the *C. albicans* genome that is available to the research community (<http://www.candidagenome.org/CommunityNews.shtml>). This clone has the Tn7-*UAU1* transposon inserted within the open reading frame of *C. albicans* *KAR2* at approximately position 541 bp (Davis et al. 2002). Prior to transformation into yeast containing one *KAR2* allele under control of the *MET3* promoter, the plasmid was digested with *Not*I to generate a linear ~10 kb fragment, which contained the *UAU1* cassette and *KAR2* flanking homology.

Southern blot analysis was performed as previously described by Sambrook and Russell (2001). Genomic DNA was isolated from the parental strain, BWP17, as well as from each transformant. A total of 1 µg of genomic DNA from each transformant was digested with *Alw*NI. The template DNA for the probe was a PCR amplified 1,529 bp fragment, starting 38 bp after the stop codon of the *KAR2* gene, which used primers F-CaKar2-Arg4-KpnI (5'-CGTAGGTACCGCATTC CAATGTTTAGTTCTC-3') and R-CaKar2-Arg4-KpnI (5'-CGTAGGTACCATGCGAATGGTCATGGTTGT-3') designed using the CGD. The fragment was digested with *Sna*BI, resolved on an agarose gel, and the 1,234 bp piece was gel purified, followed by radiolabeling with the Prime-a-Gene Labeling System (Promega, Madison, WI) and random oligonucleotide primers.

### Complementation of *S. cerevisiae* *KAR2* in vivo

Strains MMY101 and MMY102 were grown for 7 days at 23°C on SC-URA plates (0.67% yeast nitrogen base + (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2% glucose, all supplements except uracil), while strains RSY607 and RSY586 were grown for 3 days at 23°C on YPD plates. Two colonies from each plate were suspended in 200 µL of sterile water. The OD<sub>600</sub> for each of the four cultures was determined and normalized by adding the appropriate amounts of sterile water. Tenfold serial dilutions of each culture were prepared using sterile water, and 5 µL of each dilution were spotted onto each of two YPD plates. One plate was incubated at 23°C and the other was incubated at 37°C. Digital photographs were taken of the plates after 2 days of incubation at their respective temperatures followed by one additional day at 23°C.

In order to generate a *S. cerevisiae* strain containing only the *C. albicans* *KAR2* gene, tetrad dissection was utilized. The hemizygous diploid *S. cerevisiae* strain YJ034W BY4743 (ATCC #4021388) was used, which contains one wild-type allele of the *KAR2* gene and one *KAR2* allele that has been replaced with the *kanMX* gene, conferring resistance to G418 (Wach et al. 1994). This strain was transformed with plasmid pCaKAR2-BX and plated on SC-URA. After screening positive transformants for the presence of pCaKAR2-BX, these cells, named strain MMY105, were sporulated and subjected to standard tetrad dissection on

YPD plates (Rose et al. 1990). The plates were then incubated at 30°C until the spores grew into visible colonies.

Cells from the four colonies resulting from the growth of the four spores of one tetrad were streaked onto SC-URA and YPD + 100 µg/mL G418 (USB, Cleveland, OH). These plates were incubated at 30°C for 2 days. Cells from the same four colonies were also grown overnight in liquid YPD at 30°C. The cells were then harvested from 750 µL of the culture and extracts were prepared by suspending the cells in 20 µL of SDS-PAGE sample buffer (65 mM Tris, pH 6.8, 2 mM β-mercaptoethanol, 2% SDS, 0.25 mg/mL bromophenol blue, 10% glycerol). The samples were then heated at 70°C for 10 min followed by the addition of 0.12 g of 425–600 µm micron glass beads (Sigma–Aldrich). Finally, the samples were agitated with a vortex mixer for 1 min and extracts were frozen at –20°C.

Extracts were then diluted 1:10 and run on a 15% SDS-PAGE gel. The proteins were transferred to Protran BA85 nitrocellulose (Schleicher and Schuell, Keene, NH), blocked in 2% milk in TBST (50 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween-20) and cut just below the 60 kD Benchmark molecular weight marker (Invitrogen, Carlsbad, CA). The top portion of the blot was incubated overnight at 4°C with a 1:3,000 dilution of a polyclonal antibody that was generated against the *S. cerevisiae* Kar2 protein (Brodsky and Schekman 1993). The bottom portion of the blot was incubated in the same manner, but with an antibody generated against the *S. cerevisiae* Sec61 protein (Stirling et al. 1992). The blots were then washed with TBST and incubated for 1 h at 23°C in a 1:3,000 dilution of HRP-conjugated α rabbit IgG secondary antibody (Invitrogen). After adding chemiluminescence reagents (Pierce Biotechnology, Rockford, IL), the blots were exposed to film and developed.

### Microsome preparation and in vitro translocation reactions

ER microsomes were prepared from *S. cerevisiae* strains MMY101, MMY102, MMY108, RSY607 and RSY586 after the cells were grown in their respective media (SC-URA for the MMY strains and YPD for the RSY strains) to an OD<sub>600</sub> of ~2.0. *C. albicans* strain BPY116 cells were grown in YNB + His medium and then used to inoculate fresh YNB + His and YNB + His + 2.5 mM Met, Cys media to an OD<sub>600</sub> of 0.2. Microsomes were prepared from these cells after 6 and 8 h of growth at 30°C. *S. cerevisiae* microsomes were purified as previously described (Brodsky et al. 1993). Microsomes isolated from *C. albicans* cells were prepared in the same manner except that commercial zymolyase (100T; US Biologicals, Swampscott, MA) was used at a concentration of 0.3 mg/mL of suspended cells. Microsomes were then used in in vitro post-translational translocation assays, which were performed as described (Morrow and Brodsky 2005; Brodsky et al. 1993, Rothblatt et al. 1989), except that translocation reactions were incubated at 20°C for 40 min, cytosol was added to 5 mg of protein/mL and the protein pellets were washed twice with acetone after TCA precipitation. The substrate used in these reactions was in vitro transcribed/translated *S. cerevisiae* 3Δg-ppaf, which is lacking the three N-linked glycosylation sites (McCracken and Brodsky 1996) and was prepared using a rabbit reticulocyte lysate system (Promega), and L-[<sup>35</sup>S] methionine (Perkin Elmer, Waltham, MA). Translocation assays using microsomes prepared from *C. albicans* cells contained cytosol isolated from wild-type *C. albicans* cells (SC5314), prepared as described for *S. cerevisiae* (Sorger and Pelham 1987). The amounts of all microsomes used in each translocation reaction were normalized by adjusting the absorbance at 280 nm of a 1:100 dilution in 2% SDS with buffer 88 (20 mM HEPES, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 250 mM sorbitol). Translocation efficiencies were calculated by dividing the amount of protease-protected paf in the trypsin treated reaction by the total of the 3Δg-ppaf and paf in the untreated reaction. Standard error of the mean (SE) was then calculated for translocation reactions using each type of microsome.



## Quantitative real-time PCR

BPY116 *C. albicans* cells were grown over night in YNB + His and then diluted to an OD<sub>600</sub> of 0.2 in either YNB + His or YNB + His + 2.5 mM Met and Cys. The cells were grown for 8 h at 30°C and 3 ODs of cells were then harvested. Total RNA was purified from these cells using the Masterpure Yeast RNA Purification Kit (Epicentre Biotechnologies, Madison, WI) followed by DNase I treatment using a Turbo DNA-free kit (Applied Biosystems, Austin, TX). Complementary DNA was made using 500 ng of total RNA, Omniscript reverse transcriptase (Qiagen) and oligo dT primers (Applied Biosystems). Quantitative PCR was then performed using the SYBR green IQ mastermix (Bio-Rad, Hercules, CA), an iCycler thermocycler (Bio-Rad) and *KAR2*-specific primers fCa-Kar2realtime1 (5'-CCAATCACAGCAAAATTATACGGTGG-3') and rCaKar2realtime1 (5'-CATCGTGATCGAATTCATCATCTGAA-3'), designed using the CGD. Real-time PCR primers were designed to amplify ~100 bp products within 300 bp of the translational stop codon for each respective gene. Primers amplifying actin (*ACT1*) and alpha-tubulin (*TUB1*) cDNA were used for normalization controls. Data were analyzed as normalized fold expression as described (Livak and Schmittgen 2001). Results presented are the mean and standard deviations of two biological replicates consisting of six total technical replicates for each gene and condition, except where indicated otherwise.

## Results

### The *C. albicans* *KAR2* gene is essential

The requirement of the *C. albicans* *KAR2* gene for yeast survival has not been investigated, although the *S. cerevisiae* *KAR2* gene is essential (Rose et al. 1989; Normington et al. 1989). Therefore, a conditional null mutant for the *C. albicans* *KAR2* gene was generated by placing one allele under control of the tightly controlled *C. albicans* *MET3* promoter (Care et al. 1999) before disrupting the other allele with a *UAU1* cassette (Enloe et al. 2000). Southern blot analysis confirmed that strain BPY116 contains one intact *KAR2* allele, which is controlled by the *MET3* promoter (Fig. 1).

To determine if *C. albicans* *KAR2* is essential, the *MET3* promoter was used to repress the transcription of the lone functional *KAR2* allele in strain BPY116 through the addition of 2.5 mM methionine and cysteine to the growth media (Care et al. 1999). Two independent isolates (BPY116a and BPY116b) were plated on the appropriate selective media either lacking (Fig. 2a) or containing 2.5 mM methionine and cysteine (Fig. 2b). After incubation at 30°C, neither of the conditional null mutants were viable in the presence of 2.5 mM methionine and cysteine (Fig. 2b). These results suggest that a functional copy of the *KAR2* gene is essential in *C. albicans*.

### Ca*KAR2* rescues the growth of a *kar2* *Saccharomyces cerevisiae* mutant strain

To better understand the function of the *C. albicans* Kar2 protein, we first determined if the Ca*KAR2* gene can rescue the temperature-sensitive growth phenotype observed for a *S. cerevisiae* strain containing the *kar2-159* mutation, which is inviable at 37°C (Fig. 3; Vogel et al. 1990). This strain is defective for both the translocation of secretory proteins into the ER lumen (Vogel et al. 1990) and during the export of soluble secretory proteins back into the cytosol for degradation (Nishikawa et al. 2001).

A *S. cerevisiae* *kar2-159* strain (RSY586) was transformed with plasmid pCa*KAR2*-BX, containing the *C. albicans* *KAR2* gene under control of the *CUP1* promoter, and with the control plasmid (pYEX-BX), generating strains MMY102 and MMY101, respectively. An isogenic wild-type stain, RSY607, was also examined. As seen in Fig. 3, all four strains grow well at the permissive temperature (22°C). However, the presence of the Ca*KAR2*

gene, which encodes no CUG codons, rescues growth at the nonpermissive temperature (Fig. 3; MMY102), while the vector-only control continues to display a temperature-sensitive phenotype (Fig. 3; MMY101). These results indicate that the *CaKAR2* gene partially rescues the *kar2-159* thermosensitive growth defect in *S. cerevisiae*.

### **CaKAR2 can serve as the sole copy of the *KAR2* gene in *S. cerevisiae***

Since it is possible that the mutant form of *S. cerevisiae* Kar2p in strain MMY102 could work synergistically with *C. albicans* Kar2p to allow the cells to live at the non-permissive temperature, a *S. cerevisiae* strain containing the *C. albicans* *KAR2* gene as the sole copy of the gene was produced. A classical tetrad dissection approach was employed to generate such a strain from a *KAR2* hemizygous diploid *S. cerevisiae* strain (YJ034W BY4743) containing one wild-type *KAR2* allele and a *KAR2* allele that is deleted with the *kanMX* gene, conveying resistance to G418 (this strain does not contain the *kar2-159* mutation). This strain was transformed with plasmid pCaKAR2-BX, containing the wild-type *C. albicans* *KAR2* gene, generating strain MMY105, which was then sporulated. Spores obtained after dissection resulted in four equally sized colonies (Fig. 4a), while the diploid parent strain lacking the *CaKAR2* plasmid resulted in only two colonies (data not shown). Since *ScKAR2* is an essential gene in *S. cerevisiae* (Rose et al. 1989; Normington et al. 1989), these results indicate that the *C. albicans* *KAR2* gene can replace the essential functions of *KAR2* in *S. cerevisiae*.

To establish that two of the four colonies obtained during tetrad dissection were comprised of cells containing only the *CaKAR2* gene, cells from each of the four spores from a single tetrad were streaked onto both SC-URA and YPD + 100  $\mu$ g/mL G418. As can be seen in Fig. 4b, all four of the colonies contained cells that could grow in the absence of uracil in the growth media, suggesting that all four contained the plasmid harboring the *C. albicans* *KAR2* gene. In addition, only two of the four colonies could grow in the presence of 100  $\mu$ g/mL G418 (Fig. 4c; spores 1 and 2), which indicates that the cells from these two spores lack the *S. cerevisiae* copy of *KAR2* (Fig. 4c).

ScKar2 protein expression was also assessed in cells derived from the four spores to verify that only two spores were expressing ScKar2p. Cell extracts were prepared from cells derived from all four spores. These extracts were then analyzed by western blot using an antibody generated against *S. cerevisiae* Kar2p (Brodsky and Schekman 1993). As can be seen in Fig. 4d, while all four lanes had comparable amounts of protein, as indicated by similar amounts of  $\alpha$ ScSec61p immunoreactivity, only the cells derived from the spores that were susceptible to G418 possessed ScKar2p (spores 3 and 4). These results suggest that the *S. cerevisiae* Kar2 protein is absent from cells derived from two of the four spores dissected from strain MMY105, and that the anti-ScKar2p antiserum does not cross react with the *C. albicans* Kar2 protein.

### **CaKar2p supports the translocation of a presecretory protein into ER-derived microsomes from *S. cerevisiae***

The data presented above support the idea that CaKar2p can functionally replace Kar2p from *S. cerevisiae*. To establish this fact at the level of CaKar2p's ability to translocate presecretory proteins into the ER, a well-established in vitro translocation assay was employed (Morrow and Brodsky 2005; Morrow and Brodsky 2001; Brodsky et al. 1993). Briefly, yeast-derived ER microsomes were combined with in vitro translated <sup>35</sup>S-labeled prepro alpha factor lacking the three N-linked glycosylation sites (3 $\Delta$ g-ppaf; McCracken and Brodsky 1996), purified yeast cytosol, and an ATP regenerating system. After incubation, the reactions were separated into three tubes. One tube remained untreated while one was treated with trypsin, and the other with trypsin and Triton X-100. Upon successful

translocation, the 3 $\Delta$ g–ppaf signal sequence is cleaved from this ~18.6 kD secreted yeast mating pheromone, resulting in the 18 kD pro-alpha factor (paf) species, which is contained within the microsomes and protected from the exogenously added protease. The Triton X-100 treated reaction serves as a control to show that the protease-protected paf is dependant on an intact membrane and is not simply protease resistant due to aggregation. It should be noted that *S. cerevisiae* 3 $\Delta$ g–ppaf is prone to aggregation, which generates a subpopulation of protease resistant 3 $\Delta$ g–ppaf (Brodsky and Schekman 1993).

ER microsomes were purified from *S. cerevisiae* strain MMY102, which contains the *Sckar2-159* allele in addition to the plasmid expressing the *C. albicans* *KAR2* gene (described above). Microsomes were also purified from an isogenic wild-type *S. cerevisiae* strain (RSY607) and RSY586, which contains the *kar2-159* allele but lacks the *CaKAR2*-expressing plasmid. ER microsomes prepared from RSY586 have been shown to be defective in in vitro translocation reactions performed at 20°C (Brodsky et al. 1995, 1993; Sanders et al. 1992). All three microsome preparations were then used in in vitro translocation assays as described above. Figure 5a shows that microsomes isolated from wild-type yeast cells (RSY607) translocate 3 $\Delta$ g–ppaf, forming trypsin-protected paf (lane 2), while the *kar2-159* mutant microsomes lacking CaKar2p (RSY586) are defective for this process. Microsomes prepared from wild-type yeast converted 22.5% (SE =  $\pm$ 2.7%, *n* = 4) of the 3 $\Delta$ g–ppaf to protease-protected paf, while microsomes prepared from the *kar2-159* mutant strain only converted 7.1% (SE =  $\pm$ 1.0%, *n* = 4) of the 3 $\Delta$ g–ppaf to protease-protected paf. In contrast, microsomes prepared from strain MMY102, containing both the mutant *S. cerevisiae* and wild-type *C. albicans* Kar2 proteins, translocate 3 $\Delta$ g–ppaf to levels approaching that of wild type (16.5%, SE =  $\pm$ 1.6%, *n* = 4; Fig. 5a). These results indicate that CaKar2p replaces the function of the *S. cerevisiae* Kar2 protein during post-translational translocation.

If the *C. albicans* Kar2 protein functions similarly to the *S. cerevisiae* Kar2 protein during translocation, then microsomes prepared from a *S. cerevisiae* strain containing only the *C. albicans* *KAR2* gene should also be able to translocate 3 $\Delta$ g–ppaf. To this end, *S. cerevisiae* strain MMY108, containing only CaKar2p (from the tetrad dissection), was grown to log phase and microsomes were prepared. These microsomes, as well as microsomes prepared from wild type and the *kar2-159* mutant, which serve as positive and negative controls, respectively, were then used in translocation assays as described above. As shown in Fig. 5b, microsomes containing *C. albicans* Kar2p as the only version of the protein (MMY108) translocate 3 $\Delta$ g–ppaf as effectively as those containing wild-type *S. cerevisiae* Kar2p. Quantitation of the protease-protected paf suggests that 23.3% (SE =  $\pm$ 2.3%, *n* = 16) and 23.5% (SE =  $\pm$ 3.1%, *n* = 16) of the 3 $\Delta$ g–ppaf was converted to protease-protected paf in reactions containing microsomes from wild type and CaKar2p-containing cells, respectively. These results suggest that the *C. albicans* Kar2 protein functions much like the *S. cerevisiae* version of the protein, and can replace its function during the translocation of presecretory proteins into the ER.

### CaKar2p is involved in the process of ER translocation in *C. albicans*

In order to determine if Kar2p plays a role during the translocation of presecretory proteins into the ER in *C. albicans*, we performed in vitro translocation assays using ER microsomes prepared from *C. albicans* strain BPY116 either expressing or not expressing CaKar2p. To verify that strain BPY116 expresses *KAR2* mRNA when grown in the absence of methionine, but not when grown in the presence of methionine, a qRT-PCR experiment was performed. A concentration of 2.5 mM methionine and cysteine was used in these experiments since this concentration did not have an affect on the growth of wild-type *C. albicans* cells (data not shown). BPY116 cells grown in the presence of 2.5 mM methionine and cysteine for 6 and 8 h express ~14-fold (SD =  $\pm$ 5.4) and over 1,000-fold (2,422-fold, SD



=  $\pm 1,227$ ) less *KAR2* mRNA, respectively, as compared to BPY116 cells grown for 8 h in the absence of methionine and cysteine (Fig. 6a). These results strongly suggest that the inducible promoter in strain BPY116 functions as expected with regard to *KAR2* mRNA accumulation.

*Candida albicans* strain BPY116 was grown in medium lacking methionine and cysteine and then used to inoculate two cultures, one lacking methionine and cysteine and the other containing 2.5 mM concentrations of the two amino acids. After 6 and 8 h of growth, the cells were harvested and ER microsomes were isolated. These microsomes were then used in in vitro translocation assays as described above, but using cytosol isolated from wild-type *C. albicans* cells. As can be seen in Fig. 6b, after 8 h of growth, microsomes prepared from *C. albicans* cells expressing Kar2p (no Met/Cys) displayed a significantly higher translocation efficiency (36.3%, SE =  $\pm 1.96\%$ ,  $n = 18$ ) than those prepared from cells expressing reduced amounts of *KAR2* mRNA (2.5 mM Met/Cys; 10.0%, SE =  $\pm 0.54\%$ ,  $n = 18$ ). In addition, the efficiency of translocation into microsomes prepared from cells expressing reduced amounts of *KAR2* mRNA decreased over the amount of time grown in medium containing methionine and cysteine (Fig. 6c), suggesting that Kar2p turnover results in decreased translocation activity. Together, these data support the conclusion that Kar2p is required for the translocation of presecretory proteins into the *C. albicans* ER.

## Discussion

We have determined that the *C. albicans* *KAR2* gene is essential and functions during the translocation of proteins into the endoplasmic reticulum, indicating the importance of the encoded Hsp70 molecular chaperone protein in *C. albicans*. It is probable that the absence of Kar2p function during the process of secretory protein transport into the ER is the reason for inviability under the conditions used in this study, a suggestion that is supported by the fact that reduced Kar2p expression results in a translocation defect in *C. albicans* (Fig. 6b, c). This is likely because translocation into the ER is the first committed step of the secretory pathway, which is an essential process for protein targeting in the cell. Since ScKar2p has also been shown to play an essential role during protein folding in *S. cerevisiae* (Simons et al. 1995), it is also possible that the deletion of *CaKAR2* results in inviability due to a defect in protein folding.

As well as its essential roles during translocation and protein folding, it is possible that the *C. albicans* Kar2 protein may also display functional overlap with other non-essential roles that Kar2p plays in *S. cerevisiae*, such as during the process of ER-associated degradation (ERAD, Nishikawa et al. 2005; 2001; Kabani et al. 2003; Plemper et al. 1997) and karyogamy (Ng and Walter 1996; Latterich and Schekman 1994; Rose et al. 1989; Normington et al. 1989). Interestingly, a recent study has identified that *C. albicans* Jem1p, which is a co-chaperone protein that interacts with Kar2p and functions during ERAD and karyogamy, can complement karyogamy and ERAD defects in *S. cerevisiae* (Makio et al. 2008). Given the facts that our study shows that CaKar2p can replace the essential functions of ScKar2p, and that Makio et al. (2008) showed that CaJem1p can effectively interact with ScKar2p during ERAD and karyogamy, together these results suggest that Kar2p and Jem1p may interact in *C. albicans* as they do in *S. cerevisiae* during ERAD. In support of this hypothesis, Wimalasena et al. (2008) have shown that some of the machinery involved in *C. albicans* ERAD is regulated similar to that of *S. cerevisiae*.

In addition to our in vivo data, we also determined that the *C. albicans* Kar2 protein functions in place of *S. cerevisiae* Kar2p during the translocation of a presecretory protein into the ER lumen. In fact, translocation efficiencies using *S. cerevisiae* microsomes containing only CaKar2p were nearly identical to the efficiencies obtained when using

microsomes containing only wild-type ScKar2p. These results suggest that *C. albicans* Kar2 protein can completely replace the function of the *S. cerevisiae* Kar2 protein during translocation. However, in translocation reactions using microsomes containing both a mutant form of ScKar2p in addition to wild-type *C. albicans* Kar2p, translocation efficiencies were approximately 73% of microsomes containing only wild-type ScKar2p (16.5% for CaKar2p + mutant ScKar2p and 22.5% for wild-type ScKar2p). In these translocation reactions, it is possible that the presence of a mutant form of *S. cerevisiae* Kar2p inhibits the complete rescue of translocation activity by *C. albicans* Kar2p. This could be due to the fact that the mutant form of the *S. cerevisiae* protein may engage in normal complex formation with ScSec61p, ScSec63p and other proteins, thus sequestering these other factors from forming functional complexes with the *C. albicans* Kar2 protein. Since Kar2p must associate with Sec63p and Sec61p to perform its many roles during the translocation of proteins into the ER (Alder et al. 2005; Young et al. 2001; McClellan et al. 1998; Matlack et al. 1997; Lyman and Schekman 1995; Sanders et al. 1992), it is possible that this sequestration could inhibit the translocation process in these microsomes.

Importantly, we were able to successfully use an in vitro translocation assay to show that *C. albicans* microsomes isolated from a strain expressing reduced amounts of Kar2p display a translocation defect when compared to microsomes containing normal levels of Kar2p (Fig. 6). This is the first example of the use of an in vitro assay to study ER translocation using components derived from *C. albicans*, which provides a very useful system for studying the roles of other proteins involved in ER translocation. Our translocation assays using *C. albicans* cytosol and ER microsomes isolated from a *C. albicans* strain expressing Kar2p from the *MET3* promoter displayed translocation efficiencies (~36%; Fig 6c, no met/cys) that surpass translocation efficiencies using components derived from wild-type *S. cerevisiae* (~23%; see Figs. 5a, b, RSY607), indicating that this assay functions similarly with *C. albicans* components. It is possible that this increased efficiency is due to the enhanced *KAR2* expression from the *MET3* promoter used in strain BPY116. In vitro translocation assays using ER-derived microsomes and reconstituted proteoliposomes (Sanders et al. 1992) have been used to extensively investigate ER translocation processes in *S. cerevisiae* including the establishment of the minimal components necessary for post-translational translocation (Panzner et al. 1995), the components that interact with the signal sequence of secretory proteins (Plath et al. 1998), and specific chaperone interactions (Brodsky et al. 1993). Additionally, in vitro translocation reactions are the first step in cell-free ERAD assays that have been used to dissect the mechanisms of this process in *S. cerevisiae* (Lee et al. 2004, 2005; McCracken and Brodsky 1996). The development and use of this translocation assay using *C. albicans* components may assist in establishing a deeper understanding of how protein import into the ER and ERAD occur in this human pathogen.

Finally, while the classic secretory pathway appears to be relatively conserved in eukaryotes, alternative secretory pathways are used in *C. albicans* (reviewed in Fonzi 2009 and Nombela et al. 2006). In addition, the *C. albicans* Sec61 protein appears to be unable to functionally replace its orthologue in *S. cerevisiae*, despite being 76% similar and codon-corrected for expression in *S. cerevisiae* (de la Rosa et al. 2004b). The Sec20 protein in *C. albicans* has also been suggested to be unable to complement its orthologue in *S. cerevisiae* (Weber et al. 2001). While there are multiple possible explanations as to why these *C. albicans* proteins do not complement the functions of their *S. cerevisiae* homologues, these studies suggest that while the secretory pathway is highly conserved in eukaryotic systems, specific differences may exist in *C. albicans*. Since the classic secretory pathway is essential for viability and virulence of this organism, differences in the function(s) of proteins involved in this pathway may serve as potential future chemotherapeutic targets for preventing and treating candidiasis. It will be interesting to examine the direct role of the secretory pathway on *C. albicans* virulence in future studies.

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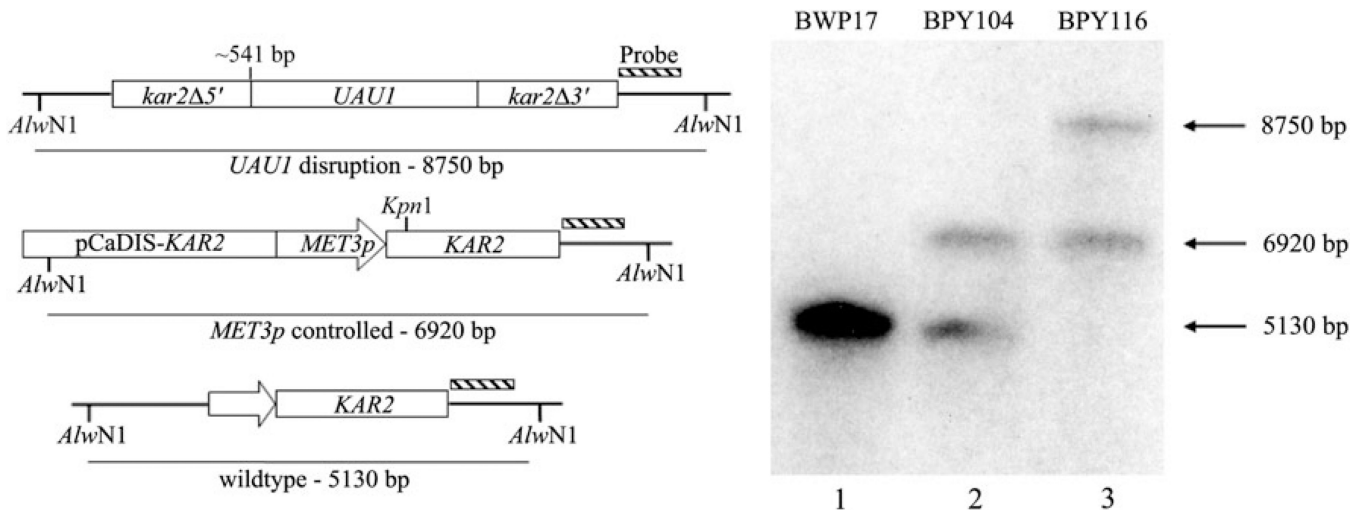
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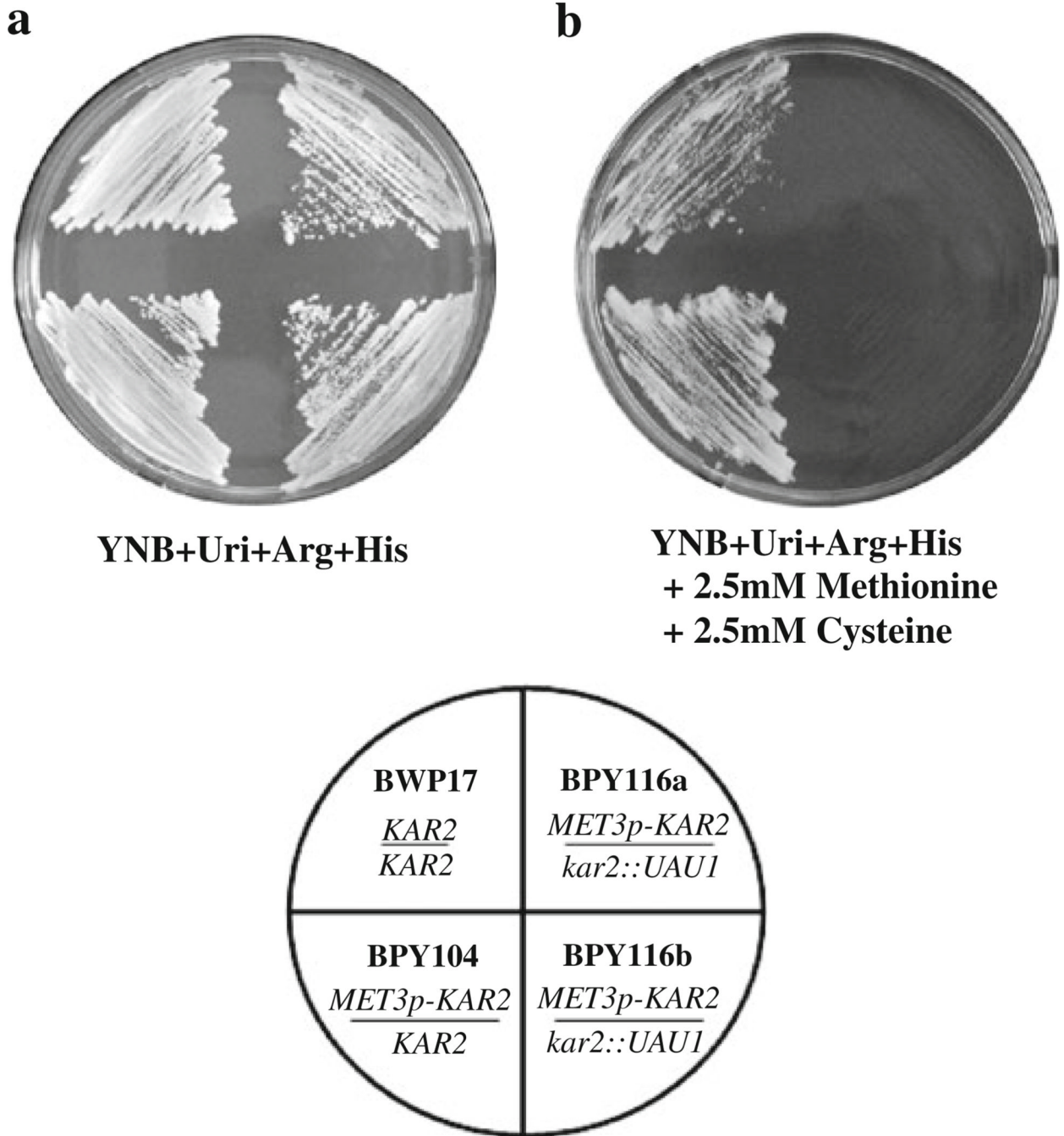


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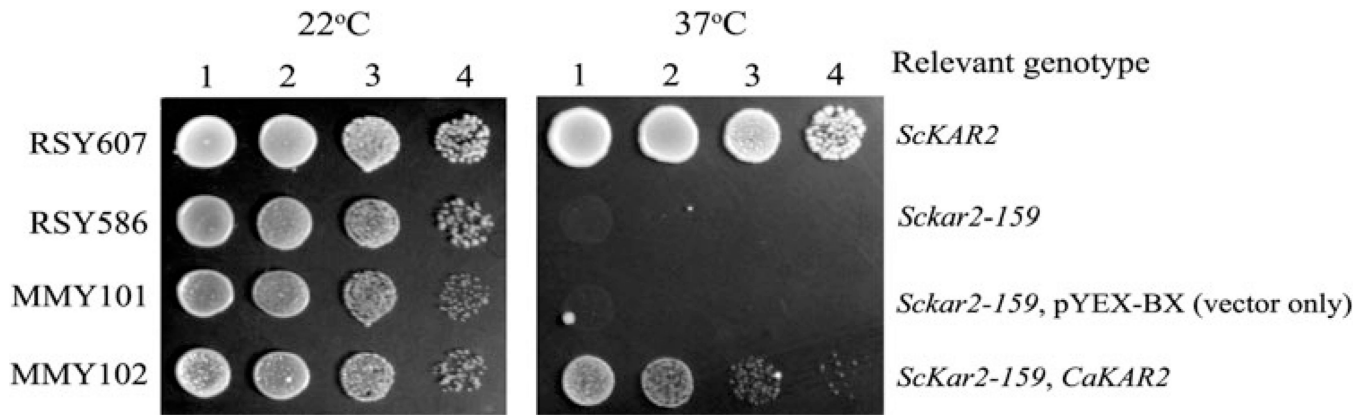


**Fig. 1.**

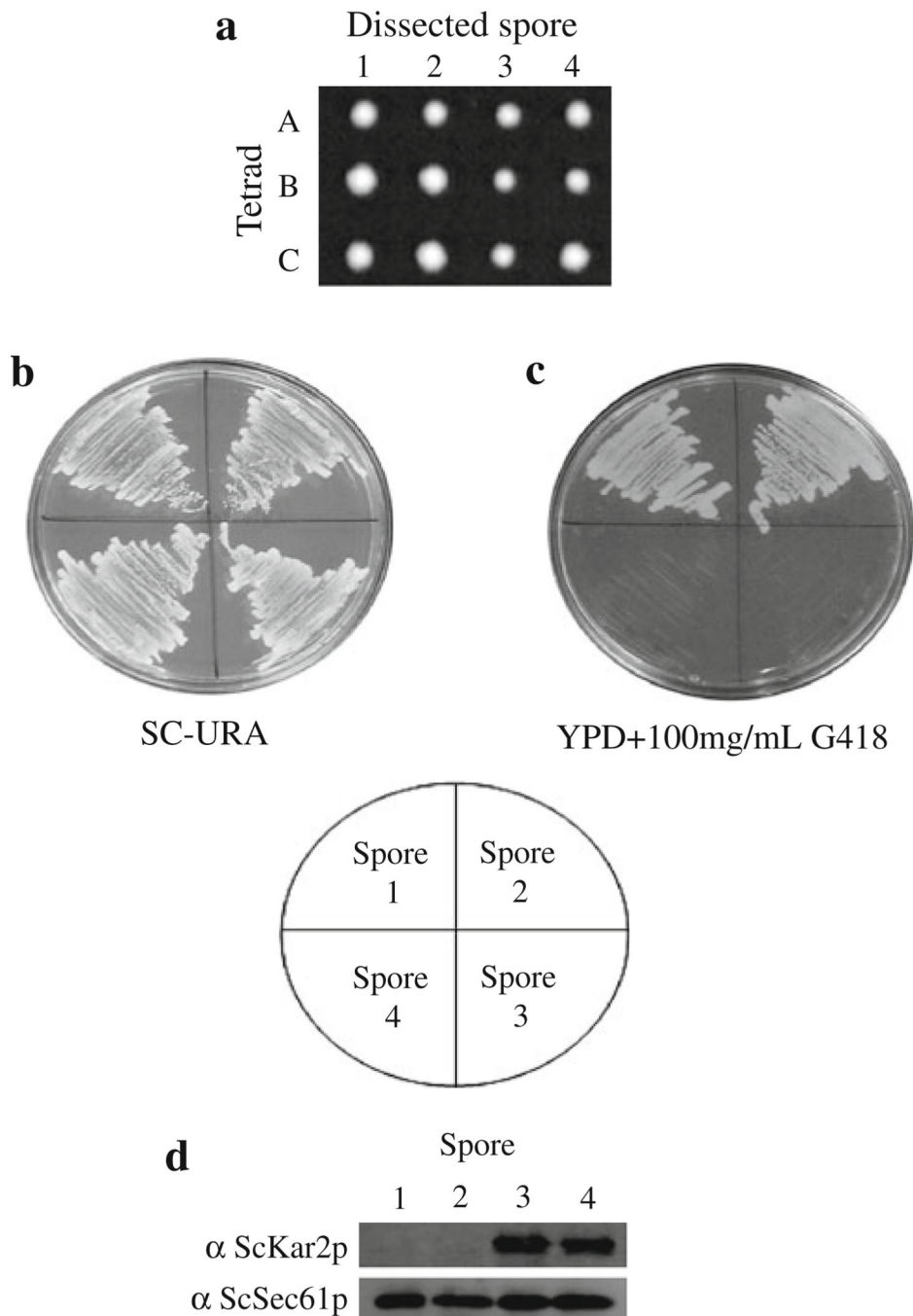
Confirmation of *KAR2* conditional null mutant in *Candida albicans* through Southern blot analysis. The diagram on the left depicts the three expected restriction fragments after the digestion of genomic DNAs with *AlwNI*. When probed, BWP17 (lane 1) results in a single band, representing two wild-type *KAR2* alleles. Placing one *KAR2* allele under control of the *MET3* promoter (BPY104; lane 2) increases the size of the restriction fragment of one allele so that two bands are evident. The *UAU1* disruption of the remaining wild-type *KAR2* allele (BPY116; lane 3) corresponds to the emergence of the largest restriction fragment, the continued presence of the *MET3* controlled allele, and the disappearance of the band corresponding to the wild-type size

**Fig. 2.**

The *Candida albicans* *KAR2* gene is essential. Strains BWP17 (*KAR2/KAR2*), BPY104 (*MET3p-KAR2/KAR2*), BPY116a, and BPY116b (both *MET3p-KAR2/kar2::UAU1*) were streaked onto plates under conditions in which the *MET3p*-controlled *KAR2* gene was either induced in medium lacking methionine and cysteine (**a**) or repressed in medium containing 2.5 mM methionine and 2.5 mM cysteine (**b**). Both plates were grown at 30°C for ~48 h. The conditional null mutants (BPY116a and BPY116b) are inviable when *KAR2* expression is repressed by the *MET3* promoter (**b**)

**Fig. 3.**

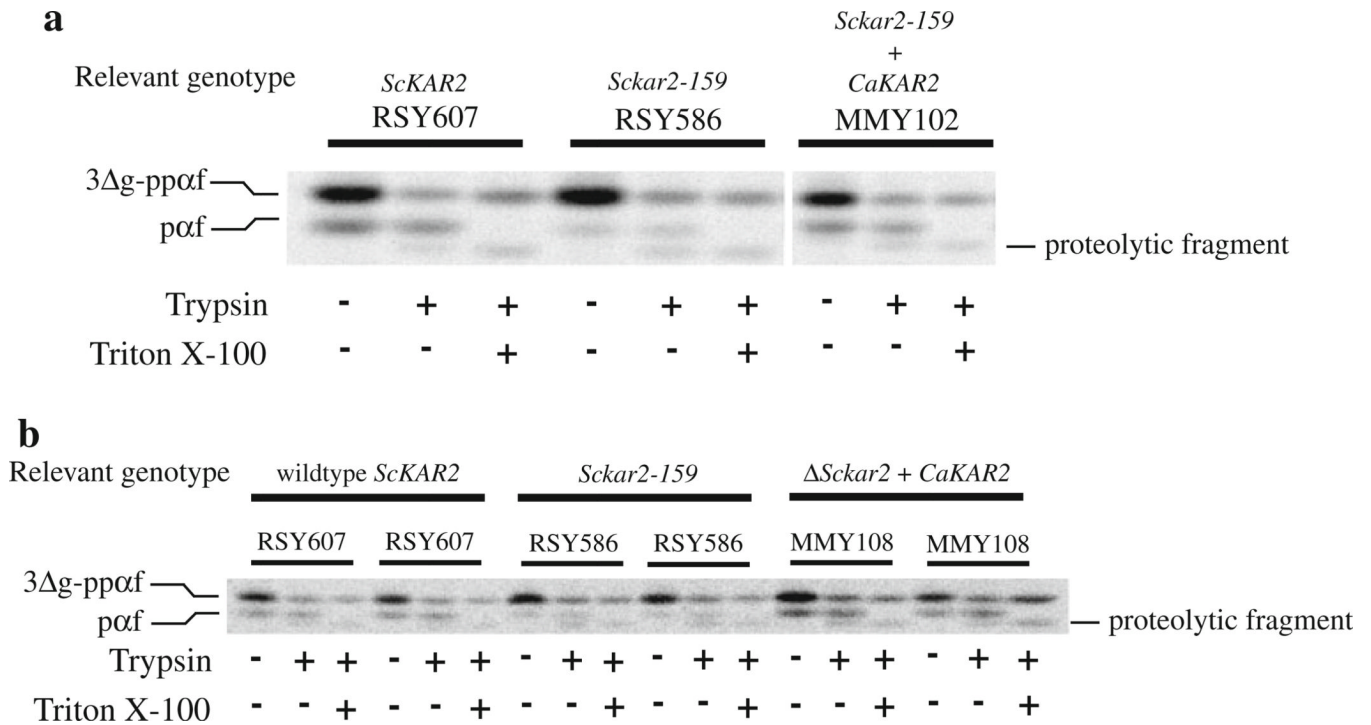
The *C. albicans* *KAR2* gene can rescue a temperature-sensitive allele of the *S. cerevisiae* *kar2* gene. A *S. cerevisiae* strain containing the *kar2-159* mutation (RSY586) was transformed with a plasmid expressing the *C. albicans* *KAR2* gene, generating strain MMY102. Liquid cultures of these cells were grown in appropriate media overnight along with a wild-type *S. cerevisiae* strain (RSY607) and strain MMY101, which is RSY586 containing the empty vector. The cultures were normalized to a common OD<sub>600</sub>, and 5 µl of each culture (1) and tenfold serial dilutions (2–4) were then spotted onto two YPD plates. One plate was incubated at 22°C and the other at 37°C for ~48 h



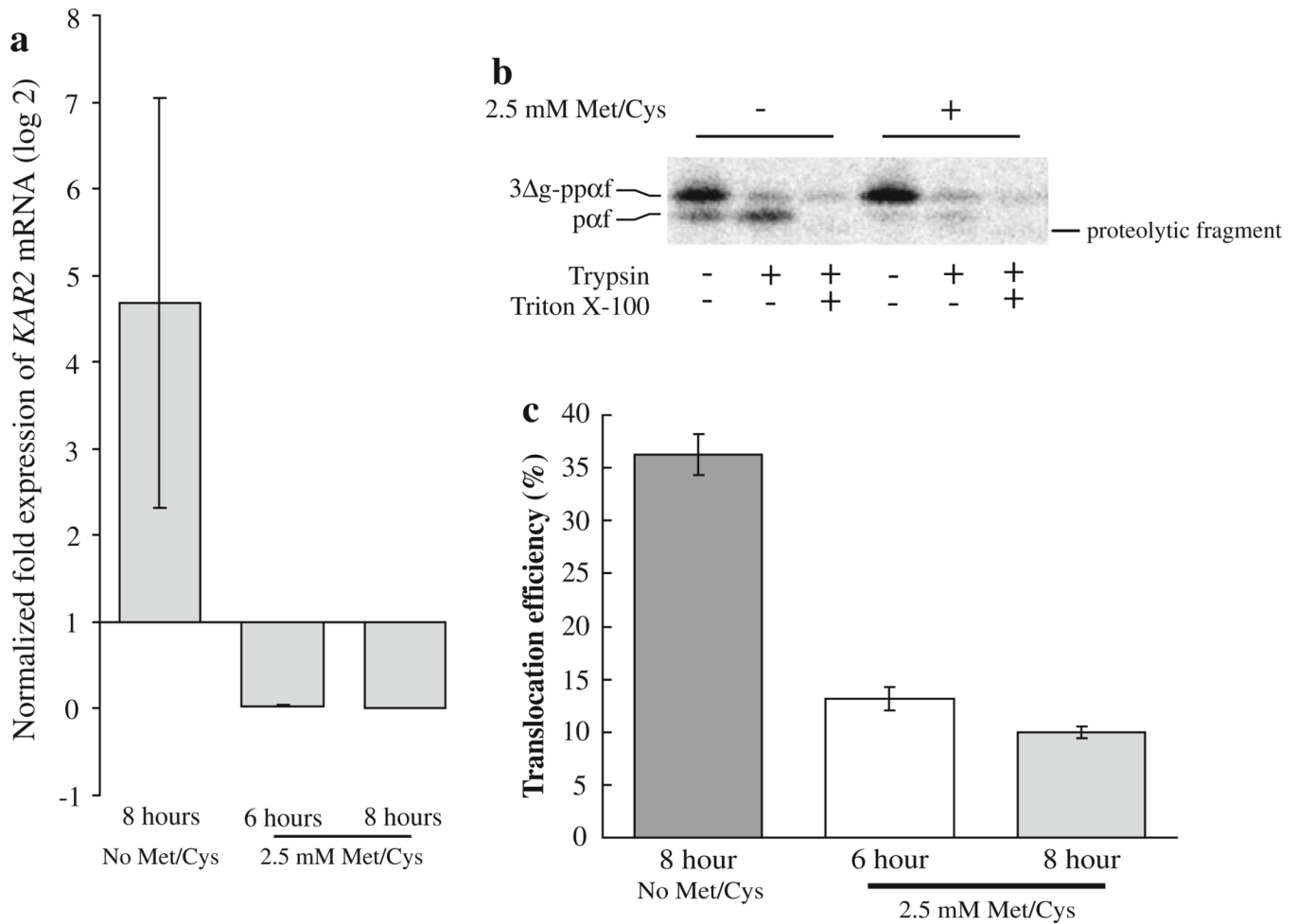
**Fig. 4.** The *C. albicans* *KAR2* gene can serve as the sole copy of this essential gene in *S. cerevisiae*. Hemizygous diploid *S. cerevisiae* strain YJ034W BY4743, which has one copy of the *ScKAR2* gene disrupted and one wild-type *ScKAR2* gene, was transformed with plasmid pCaKAR2-BX containing the *C. albicans* *KAR2* gene. The cells were then sporulated, and the four spores were dissected from each tetrad on YPD plates and incubated for ~72 h at 30°C (**a**). Three typical dissected tetrads are shown (A–C). The four spores from each tetrad resulted in normal sized colonies (1–4). To show that two of the spores from the tetrads lack the *S. cerevisiae* *KAR2* gene, colonies from all four spores of one tetrad were streaked onto a SC-URA plate (**b**) as well as a YPD plate supplemented with 100  $\mu$ g/mL G418 (**c**) and



grown at 30°C for ~48 h. Lysates were made from cells derived from each spore, and the proteins were analyzed by western blot using antibodies generated against the *S. cerevisiae* Kar2 and Sec61 proteins (**d**)

**Fig. 5.**

The *C. albicans* Kar2 protein can replace the function of *S. cerevisiae* Kar2p during translocation in vitro. ER microsomes were prepared from a wild-type *S. cerevisiae* strain (RSY607), a *kar2* mutant strain (RSY586) and strain MMY102 (**a**), which is RSY586 expressing wild-type *C. albicans* Kar2p, or MMY108 (**b**), which is a *S. cerevisiae* strain containing *C. albicans* Kar2p as the only form of Kar2p in the cells. These microsomes were used in in vitro translocation reactions using <sup>35</sup>S-labeled 3Δg-ppαf as the translocation substrate (see text). After translocation, the reactions were split equally into three tubes and either left untreated, or treated with trypsin or trypsin and Triton X-100. The proteins were then TCA precipitated and resolved by 18% SDS-PAGE in gels containing urea and subjected to phosphorimage analysis. The presence of trypsin-protected pαf indicates substrate translocation. Duplicate translocation reactions are shown in **b**

**Fig. 6.**

Kar2p functions during the translocation of a substrate protein into the *C. albicans* ER in vitro. BPY116 *C. albicans* cells were grown in YNB + His (No Met/Cys; inducing conditions) or YNB + His + 2.5 mM methionine and cysteine (repressing conditions) for 6 and 8 h. Total RNA was purified from three ODs of cells, and qRT-PCR was performed to determine the amount of *KAR2* mRNA present in the cells (a). The data represent the means of two biological replicates with a total of six technical replicates for the repressed cultures (6 h = 0.028, SD = ±0.010, 8 h = 0.0019, SD = ± 9.9 × 10<sup>-4</sup>), and the mean of three technical replicates for the induced culture (4.68, SD = ±2.37). *KAR2* mRNA levels were normalized to actin and alpha-tubulin genes in each condition, and expression levels are relative to *KAR2* expression at the time of inoculation (zero time point, expression = 1). ER microsomes were then purified from strain BPY116 after growth for 8 h in media either containing (+) or lacking (-) 2.5 mM methionine and cysteine. The microsomes were combined with *C. albicans* cytosol, <sup>35</sup>S-labeled *S. cerevisiae* 3Δg-ppαf and an ATP regenerating system. Translocation assays were performed as described, and typical reactions are shown (b). The presence of trypsin-protected pαf indicates successful translocation. Translocation reactions were also performed using microsomes prepared from strain BPY116 after growth under repressing conditions for 6 and 8 h (*n* = 15 and 18, respectively). Average translocation efficiencies were compared to translocation efficiencies into microsomes prepared from BPY116 cells grown under inducing conditions for 8 h (*n* = 18). Standard error of the means are shown as error bars (c)

Table 1

## Strains used in this study

Strain	Genotype	Reference
<i>C. albicans</i> strains		
BPY104	As BWP17, but <i>KAR2/MET3p-KAR2</i>	This study
BPY116a and BPY116b	As BWP17, but <i>kar2Δ::UAU1/MET3p-KAR2</i>	This study
BWP17	<i>Δura3::imm434/Δura3::imm434 Δhis1::hisG/Δhis1::hisG Δarg4::hisG/Δarg4::hisG</i>	Wilson et al. (1999)
NIH3172	Clinical isolate	ATCC #14053
SC5314	Clinical isolate	Gillum et al. (1984)
<i>S. cerevisiae</i> strains		
MMY101	As RSY586, but containing pYEX-BX	This study
MMY102	As RSY586, but containing pCaKAR2-BX	This study
MMY105	As YJ034W BY4743, but containing pCaKAR2-BX	This study
MMY108	<i>MATa his3Δ1 leu2Δ0 ura3 Δ0 lys2Δ Δkar2::kanMX</i> and containing pCaKAR2-BX	This study
RSY586 (MS137)	<i>MATa ura3-52 leu2-3, 112 ade2-101 kar2-159</i>	Brodsky et al. (1995)
RSY607	<i>MATa ura3-52 leu2-3, 112 pep4::URA3</i>	Brodsky et al. (1993)
YJ034W BY4743	<i>MATa/MATa his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/uraΔ0 Δkar2::kanMX/+</i>	ATCC #4021388