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Protein Arginine Methylation in *Candida albicans*: Role in Nuclear Transport[∇]

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Protein arginine methylation plays a key role in numerous eukaryotic processes, such as protein transport and signal transduction. In *Candida albicans*, two candidate protein arginine methyltransferases (PRMTs) have been identified from the genome sequencing project. Based on sequence comparison, *C. albicans* candidate PRMTs display similarity to *Saccharomyces cerevisiae* Hmt1 and Rmt2. Here we demonstrate functional homology of Hmt1 between *C. albicans* and *S. cerevisiae*: CaHmt1 supports growth of *S. cerevisiae* strains that require Hmt1, and CaHmt1 methylates Npl3, a major Hmt1 substrate, in *S. cerevisiae*. In *C. albicans* strains lacking CaHmt1, asymmetric dimethylarginine and ω -monomethylarginine levels are significantly decreased, indicating that Hmt1 is the major *C. albicans* type I PRMT1. Given the known effects of type I PRMTs on nuclear transport of RNA-binding proteins, we tested whether Hmt1 affects nuclear transport of a putative Npl3 ortholog in *C. albicans*. CaNpl3 allows partial growth of *S. cerevisiae* *npl3* Δ strains, but its arginine-glycine-rich C terminus can fully substitute for that of ScNpl3 and also directs methylation-sensitive association with ScNpl3. Expression of green fluorescent protein-tagged CaNpl3 proteins in *C. albicans* strains with and without CaHmt1 provides evidence for CaHmt1 facilitating export of CaNpl3 in this fungus. We have also identified the *C. albicans* Rmt2, a type IV fungus- and plant-specific PRMT, by amino acid analysis of an *rmt2* Δ /*rmt2* Δ strain, as well as biochemical evidence for additional cryptic PRMTs.

Methylation of nitrogen atoms within arginine residues of proteins can influence a large number of cellular processes in eukaryotes, including protein transport, transcriptional activation, pre-mRNA splicing, and signaling pathways (5, 6). Effects of arginine methylation on intracellular transport can vary among eukaryotes: methylation facilitates nuclear export of several RNA-binding proteins in *Saccharomyces cerevisiae* (21, 37) but directs nuclear import of human RNA helicase A (40). Whereas the roles of arginine methylation are diverse, many roles are linked to modulation of protein-protein interactions (4, 5).

All eukaryotic organisms examined to date share one major protein arginine methyltransferase (PRMT) (5). Although this enzyme, termed Hmt1/Rmt1 in *S. cerevisiae* and PRMT1 in humans, has been shown to be the predominant PRMT in both of these organisms (18, 41), numerous other PRMTs have been identified. The human genome encodes at least nine PRMTs and different splice variants thereof (11); *S. cerevisiae* expresses three PRMTs, i.e., Hmt1, Hsl7, and Rmt2 (18, 24, 28, 29). Methyltransferase activity has been demonstrated for all three *S. cerevisiae* enzymes (18, 28, 29, 42), but their activities differ. Hmt1 is a type I PRMT, catalyzing the formation of ω -*N*^G-monomethylarginine (ω -MMA) and asymmetric *N*^G,*N*^G-dimethylarginine (ADMA) (18). Hsl7 catalyzes formation of ω -MMA (28) and shows sequence similarity to mammalian PRMT5, which forms ω -MMA and symmetric *N*^G,*N*^G-di-

methylarginine (SDMA) (8). Rmt2 catalyzes the formation of δ -MMA, in which the internal δ nitrogen of arginine is methylated (29). Although the Rmt2 sequence is slightly similar to that of a mammalian small-molecule methyltransferase that targets guanidinoacetate (29), more closely related genes are found in fungal and plant genomes but are absent in animals.

S. cerevisiae shares different subsets of PRMT genes with other fungi, including *Schizosaccharomyces pombe*, *Candida albicans*, and *Candida glabrata*. *S. pombe* has a wide range of PRMT genes, including not only *HMT1/RMT1*, *HSL7/RMT5*, and *RMT2* orthologs but also another type I methyltransferase gene, *RMT3* (3, 31, 32, 48). Whereas *C. glabrata*, which is more closely related to *S. cerevisiae* than *C. albicans* (15), bears all three PRMT genes found in *S. cerevisiae*, the *C. albicans* genome sequencing project has revealed genes similar to *HMT1* and *RMT2* but no *HSL7* ortholog (2, 43). In contrast to *S. cerevisiae* and *S. pombe*, *Candida* species are among the four most common pathogens to cause nosocomial bloodstream infections in the United States, with *C. albicans* causing the majority of these *Candida* infections (47). Therefore, understanding the molecular mechanisms at work in *Candida* and comparing them to mechanisms in other organisms may lend clinical insights. In addition to two PRMTs, *C. albicans* and *S. cerevisiae* share many genes that encode likely Hmt1 substrates due to the presence of arginine-glycine-(RG)-rich domains, including an ortholog of the major *S. cerevisiae* mRNA-binding protein Npl3.

In this study, we present the first identification of PRMTs in *C. albicans* and demonstrate functional homology of the *HMT1* genes in this fungus and *S. cerevisiae*. Deletion of *HMT1* and

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TABLE 1. Strains used in this study

| Species and strain | Genotype | Source or reference |
|----------------------|--|---------------------|
| <i>S. cerevisiae</i> | | |
| FY23 | <i>MATa ura3-52 trp1Δ63 leu2Δ1 GAL+</i> | F. Winston |
| PSY865 | <i>MATα hmt1Δ::HIS3 ade2 ade8 ura3 leu2 his3 lys1</i> | 24 |
| PSY866 | <i>MATa ade2 ade8 ura3 leu2 his3 lys1 Δhmt1::HIS3 npl3-1 + pPS1307</i> | 24 |
| PSY814 | <i>MATα npl3Δ::HIS3 ade2 ade8 can1 ura3 leu2 his3 lys1 trp1 + YCp50-NPL3-3</i> | 23 |
| PSY1191 | <i>MATa ade2 ade8 ura3 leu2 his3 Δhmt1::HIS3 Δcbp80::HIS3 + pPS1307</i> | 37 |
| YAM533 | <i>MATα hmt1Δ::HIS3 NPL3-myc::URA3 ade2 ade8 ura3 leu2 his3 lys1</i> | 26 |
| YAM535 | <i>MATa NPL3-myc::URA3 ade2 ade8 ura3 leu2 his3 lys1</i> | 26 |
| <i>C. albicans</i> | | |
| AMC11 | <i>hmt1Δ::HIS1 ura3Δ::λimm434::URA3-IRO1 arg4::hisG his1::hisG</i> | This study |
| AMC14 | <i>hmt1Δ::ARG4 ura3Δ::λimm434 arg4::hisG his1::hisG</i> | This study |
| AMC28 | <i>hmt1Δ::ARG4:HMT1 ura3Δ::λimm434 arg4::hisG his1::hisG</i> | This study |
| AMC30 | <i>rmt2Δ::HIS1 ura3Δ::λimm434::URA3-IRO1 arg4::hisG his1::hisG</i> | This study |
| AMC35 | <i>rmt2Δ::ARG4 ura3Δ::λimm434 arg4::hisG his1::hisG</i> | This study |
| AMC36 | <i>hmt1Δ::HIS1 ura3Δ::λimm434 arg4::hisG his1::hisG</i> | This study |
| AMC46 | <i>hmt1Δ::ARG4 ura3Δ::λimm434 arg4::hisG his1::hisG</i> | This study |
| AMC47 | <i>NPL3-GFP::URA3 ura3Δ::λimm434 arg4::hisG his1::hisG</i> | This study |
| AMC48 | <i>NPL3 ura3Δ::λimm434 arg4::hisG his1::hisG</i> | This study |
| AMC49 | <i>NPL3-GFP::URA3 hmt1Δ::HIS1 ura3Δ::λimm434 arg4::hisG his1::hisG</i> | This study |
| BWP17 | <i>NPL3 hmt1Δ::ARG4 ura3Δ::λimm434 arg4::hisG his1::hisG</i> | 45 |
| MLR62 | <i>ura3Δ::λimm434 ARG4::URA3::arg4::hisG his1::hisG::pTEF1-GFP</i> | A. Mitchell |
| | <i>ura3Δ::λimm434 arg4::hisG his1::hisG</i> | |

RMT2 from the *C. albicans* genome reveals roles of Hmt1 and Rmt2 in asymmetric dimethylation and δ -monomethylation of arginine, respectively. We also show data supporting the functional homology of the RG-rich domain of Npl3 between the two fungi. Lastly, given varying effects of arginine methylation on protein transport among eukaryotic systems, we have tested the effect of deletion of *HMT1* on localization of *C. albicans* Npl3; these data support the hypothesis that Hmt1 facilitates nuclear export of Npl3 in *C. albicans*.

MATERIALS AND METHODS

Yeast strains, media, and growth conditions. The yeast strains used in this study are listed in Table 1. Oligonucleotides used in plasmid and strain construction were synthesized at Integrated DNA Technologies, Inc., and are shown in Table 2. All *S. cerevisiae* strains were grown and genetic manipulations performed as previously described (26, 27, 34). *C. albicans* strains were grown in YPD medium or in synthetic dropout (SD) media lacking appropriate supplements to select for integrated markers (34); for Uri⁻ strains, YPD and SD media were supplemented with 80 μ g/ml uridine. Generation times for *C. albicans* strains were determined by diluting overnight cultures to an optical density at 600 nm (OD₆₀₀) of 0.1 in YPD and monitoring the OD₆₀₀ every 30 min. For each strain, the log of normalized OD₆₀₀ values was graphed as a function of time, and the linear part of the graph was used to calculate generation times.

Plasmid construction. All plasmids used in this study are listed in Table 3. Plasmids expressing the *C. albicans HMT1* and *Homo sapiens PRMT1* genes under the control of *S. cerevisiae HMT1* regulatory regions were constructed as follows. *C. albicans HMT1* was amplified by PCR from CAI-1 genomic DNA (a gift of R. Wheeler) using oligonucleotides AM80 and AM81. *H. sapiens PRMT1* was amplified from pPS1302 using oligonucleotides AM79 and AM82. PCR

products were digested with NdeI and NsiI and inserted into NdeI-NsiI-digested pPS1872 to produce pAM160 (Ca*HMT1*) and pAM161 (Hs*PRMT1*), which express arginine methyltransferases with an additional MH dipeptide from *S. cerevisiae* Hmt1 at the C terminus.

The protein A (PrA)-*SCHMT1* expression plasmid pAM91 was constructed by inserting an NdeI-MscI fragment from pPS1872 into NdeI-SmaI-digested pNOPPATA. The *URA3* Ca*HMT1* plasmid pAM390 was constructed by inserting an XbaI-HindIII fragment from pAM160 into XbaI-HindIII-digested pRS316. The *C. albicans HMT1* gene and surrounding regions were cloned by PCR amplification of BWP17 genomic DNA with oligonucleotides AM113 and AM114, XhoI and SpeI digestion, and insertion into XhoI-SpeI-digested pRS315, resulting in pAM322. The *C. albicans HMT1* reconstitution plasmid (pAM385) resulted from the insertion of an XhoI-BsrBI fragment of pAM322 into XhoI-SmaI-digested pDS10 (30) followed by the elimination of an internal NcoI site by QuikChange mutagenesis (Stratagene) using oligonucleotides AM203 and AM204, resulting in a silent C-to-A mutation at the Ala196 codon.

The *C. albicans NPL3* open reading frame (ORF), as defined in assembly 19 of the *C. albicans* genome sequence, was amplified from BWP17 genomic DNA with oligonucleotides AM143 and AM144, digested with NdeI and BamHI, and inserted into NdeI-BamHI-digested pNOPPATA to create the PrA-Ca*NPL3* plasmid pAM361. Homologous recombination in *S. cerevisiae* was used to create a gene fusion between the first three domains of *S. cerevisiae NPL3* and the *C. albicans NPL3* RGG domain. First, an ApaI site was introduced into the PrA-Sc*NPL3* plasmid pPS2389 through a silent G-to-C mutation in the Gly312 codon using QuikChange mutagenesis and oligonucleotides AM135 and AM136, resulting in pAM463. A PCR fragment containing the *C. albicans NPL3* RGG domain flanked by sequences upstream and downstream of the *S. cerevisiae NPL3* RGG domain was amplified using oligonucleotides AM141 and AM142. This fragment was cotransformed into FY23 with ApaI-linearized pAM463, and plasmids were rescued from cells that grew on medium lacking leucine. After transformation into *Escherichia coli* and purification, plasmids were sequenced at

TABLE 2. Oligonucleotides used in this study

| Oligonucleotide | Sequence (5'→3') ^a | Site/mutation ^b |
|-----------------|--|----------------------------|
| AM79 | <u>cccat</u> gcacGCGCATCCGGTAGTCGGTGGAGC | NsiI+ |
| AM80 | <u>cccat</u> ATGTCTGAATCAGCTACTGATAAATCAC | NdeI+ |
| AM81 | <u>cccat</u> gcacGCGTAAAAAGTAAGTGATCCACCC | NsiI+ |
| AM82 | <u>GGC</u> catATGGAAGTGCCTGTGGCCAGGCGG | NdeI+ |
| AM113 | <u>cccact</u> agtCCCTTCATTCCGTAATTTCTGTG | SpeI+ |
| AM114 | <u>cccctc</u> gagCAGCTCCAGGGAGGGCTTCG | XhoI+ |
| AM117 | GGTGCATCAGGTGCATCATCAATAACGAC | |
| AM118 | GTGATACCATAACCAGCTCAGCCAGCTC | |
| AM124 | CCCAAACAAGATGTCTGAATCAGC | |
| AM125 | GTTCTGGTGATAAAATAGCTCTCC | |
| AM131 | GCTCTTGGTGGTACTGCTAAAAGTGCCG | |
| AM135 | GAGGCGGCTTCGGTGGG <u>cc</u> AGAGGTGGATTGG | ApaI+ |
| AM136 | CCAAATCCACCTCTGG <u>g</u> CCCACCGAAGCCGCTC | ApaI+ |
| AM141 | CAGAGGTTCTGTCATTACTGTTGAAAGAGATGACAATCCTccaccaccaagaggtagaggaggtttcagaggtag | |
| AM142 | GGCTTACCTGGTTGGTGATCTTTCACGTGGAGCATCTCTgggatcgtatgatccacctctgctgtg | |
| AM143 | <u>cccat</u> ATGGATGGTCCAGTTGAAGTTACTAAACAG | NdeI+ |
| AM144 | <u>ccgga</u> TCCATAGTACATTCCACTTCTCTCCG | BamHI+ |
| AM145 | CCAATATAGATTATATCCCAAACAAGATGTCTGAATCAGCTACTGATAAATCACAATTGTC gtttccagtcacgacgtt | |
| AM146 | GGCTTTGGGGAGAAATATAATAGGGATATACCAGTCTACACATTACGAACACCAATACTA Gctgtgaattgtgagcggata | |
| AM147 | CCATATATCAACTCCTTCCCATTTCCTTCTTATCTTTCCACCCCCACACACCCCTTATCAAT gtttccagtcacgacgtt | |
| AM148 | CTTCATAAATATTTGTCTATCAGCTCCTAATCCATTGAAAAATGAGAAAAATCCCATGAGG tgtgaattgtgagcggata | |
| AM154 | ACACAAGACATATTCCTGGGCAGC | |
| AM167 | AACAAACCCACTCATCTCATCAAAA | |
| AM170 | CTGAGCTGGCTGAGCTGGTTATGGTAT | |
| AM173 | GGATACGATCGTGATAACTCAACGACAGAGGTGGATCATACGATCGTGAAAGATCTCC AACCCGTTTTggtggtggttctaaaggtgaagaattatt | |
| AM199 | GGTCGCTTTATTTCCGTACCGAG | |
| AM203 | GACTACACTCCATTATATAAACTGCaATGGAAGAACC | NcoI- |
| AM204 | GGTTCTCCATtGCAGTTTTAATAAATGGAGTGTAGTC | NcoI- |
| AM208 | CACAGGTTGCAGAAATTTGATACCC | |
| AM213 | GGTATAGAGATGCTGGTTGG | |
| AM214 | GTGGAATGCACACTACTACTAG | |
| AM244 | GGATATGATAGAGGTGGATACGATCGTGATAACTTCAACGACAGAGGTGGATCATAACG ATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | |
| AM247 | CAAACCTGTAATCTTTTCTTAATCCCTTTGGAAACACATCCTAAACGTACTTGATTCTTA GCTGATGTAGtctagaaggaccaccttgattg | |
| AM258 | CCAAAATTGGGACAACACC | |

^a Lowercase indicates nucleotide differences from endogenous genes. Introduced or deleted restriction sites are underlined.

^b +, introduced restriction site; -, deleted restriction site.

the University of Maine, Orono, DNA sequencing facility to verify proper fusion (ScNPL3 codons 1 to 278–CaNPL3 codons 231 to 336–CCC [proline codon]–ScNPL3 codons 404 to 414).

C. albicans strain construction. A PCR-based homologous recombination strategy was used to create *C. albicans* strains lacking methyltransferase genes according to established protocols (45). PCR amplification of the *HIS1* (from pGEM-HIS1) and *ARG4* (from pRSARG4ΔSpeI) genes was performed with oligonucleotides AM145 and AM146 (*HMT1*) or AM147 and AM148 (*RMT2*). Sequential transformation of BWP17 with *HIS1* and *ARG4* PCR products to create homozygous deletion mutants was followed by reintroduction of the *URA3* gene at its endogenous locus by transformation of PstI-NotI-digested pBSK-URA (30), resulting in AMC11 and AMC30, respectively. *HMT1* was reintroduced into the *hmt1Δ/hmt1Δ* strain AMC35 by transformation of NcoI-linearized pAM385 and selection on medium lacking uridine. The *URA3* marker was removed by selection on 5-fluoroorotic acid (5-FOA) and replaced at its endogenous locus as described above, to create AMC14. The *RMT2* 3' sequences available in assembly 19 of the *C. albicans* genome sequence at the time of strain construction did not include the stop codon. To extend these sequences by inverse PCR, BWP17 genomic DNA was digested with TaqI, ligated, and digested with NsiI, and *RMT2* 3' sequences were amplified using oligonucleotides AM117 and AM118. Sequencing with AM117 added 111 nucleotides to the assembly 19 *RMT2* sequence but did not reveal the stop codon. Although *RMT2* shows “complex sequence changes in assembly 20” due to the use of a different

reference strain to fill in gaps (1), these 111 nucleotides are identical in our inverse PCR data, generated using BWP17 (a third-generation derivative of the original reference strain SC5314 [45]) and in those of assembly 20. Given these considerations, only the first 369 of 412 known codons were deleted from *RMT2*, and *URA3*, but not *RMT2*, was reintroduced into the *rmt2Δ/rmt2Δ* strain (AMC30). The heterozygous strain AMC28 serves as a positive control strain that should express Rmt2.

At each step, integration was tested by PCR using the following primer combinations: AM124/AM125 (*HMT1*), AM124/AM154 (*hmt1Δ::HIS1*), AM113/AM131 (*hmt1Δ::ARG4*), AM167/AM170 (*RMT2*), AM154/AM167 (*rmt2Δ::HIS1*), AM208/AM131 (*rmt2Δ::ARG4*), and AM213/AM214 (*URA3*). Final strains were also tested by Southern blotting: *HMT1* alleles were detected by probing DraIII-digested genomic DNA with nucleotides –622 to +36 to the *HMT1* ORF; *RMT2* alleles were detected by probing HindIII/XbaI-digested genomic DNA with a 1-kb fragment PCR fragment generated from BWP17 genomic DNA using primers AM170 and AM199.

NPL3 was tagged with the green fluorescent protein (GFP) gene in *HMT1/HMT1* and *hmt1Δ/hmt1Δ* *C. albicans* strains using established protocols (19). The *GFP-URA3* cassette was amplified from pMG1602 with oligonucleotides AM173 and AM247 and products transformed into BWP17 and AMC36. Strains that grew on medium lacking uridine were tested for Npl3-GFP expression by anti-GFP immunoblotting and fluorescence microscopy. To introduce the S340A mutation and GFP tag into *NPL3* simultaneously, oligonucleotides AM173 and

TABLE 3. Plasmids used in this study

| Plasmid | Features | Source or reference |
|-----------------------|--|---------------------|
| pAM91 | <i>CEN LEU</i> pNop-PrA- <i>SCHMT1</i> Amp ^r | This study |
| pAM160 | <i>CEN LEU2</i> <i>SCHMT1p</i> -Ca <i>HMT1</i> Amp ^r | This study |
| pAM161 | <i>CEN LEU2</i> <i>SCHMT1p</i> -Hs <i>PRMT1</i> Amp ^r | This study |
| pAM322 | <i>CEN LEU2</i> Ca <i>HMT1</i> Amp ^r | This study |
| pAM361 | <i>CEN LEU</i> pNop-PrA-Ca <i>NPL3</i> Amp ^r | This study |
| pAM362 | <i>CEN LEU</i> pNop-PrA- <i>ScNPL3</i> -Ca <i>RGG</i> Amp ^r | This study |
| pAM385 | <i>URA3</i> Ca <i>HMT1</i> Amp ^r | This study |
| pAM390 | <i>CEN URA3</i> Ca <i>HMT1</i> Amp ^r | This study |
| pAM463 | <i>CEN LEU</i> pNop-PrA- <i>ScNPL3</i> - <i>Apal</i> Amp ^r | This study |
| pBSK-URA | <i>URA3</i> Amp ^r | 30 |
| pDS10 | <i>URA3</i> Amp ^r | 30 |
| pGEMHIS1 | <i>HIS1</i> Amp ^r | 45 |
| pMG1602 | <i>GFP URA3</i> Amp ^r | 19 |
| pNOPPATA | <i>CEN LEU</i> pNop-PrA Amp ^r vector | 22 |
| pPS1302 | <i>GST</i> -Hs <i>PRMT1v2</i> Amp ^r | 35 |
| pPS1307 | <i>CEN URA3</i> <i>SCHMT1</i> Amp ^r | 24 |
| pPS1872 | <i>CEN LEU2</i> <i>SCHMT1</i> Amp ^r | 27 |
| pPS2389 | <i>CEN LEU</i> pNop-PrA- <i>ScNPL3</i> Amp ^r | 49 |
| pPS2575 | <i>CEN URA3</i> <i>SCHMT1</i> - Δ <i>antenna</i> Amp ^r | 44 |
| pRSARG4 Δ SpeI | <i>ARG4</i> Amp ^r | 45 |
| pRS315 | <i>CEN LEU2</i> Amp ^r vector | 39 |
| pRS316 | <i>CEN URA3</i> Amp ^r vector | 39 |

AM244 were used to amplify the *GFP-URA3* cassette from pMG1602 prior to transformation. The presence of the mutation and the sequence of the *NPL3-GFP* junction were confirmed by PCR amplification with oligonucleotides AM143 and AM258 and sequencing.

Protein expression and interaction studies. Protein expression in *S. cerevisiae* and *C. albicans* was tested by lysing mid-log-phase cells with glass beads in radioimmunoprecipitation buffer supplemented with protease inhibitors, using a FastPrep cell disruptor (Bio101), followed by immunoblot analysis as described previously (27). CaNpl3-GFP methylation was tested by similarly lysing mid-log-phase cells in lysis buffer (150 mM KCl, 5 mM MgCl₂, 20 mM Tris-HCl, pH 8.0) containing protease inhibitors and 0.2% Triton X-100. CaNpl3-GFP was precipitated from lysates (3 mg total protein) by incubation with anti-GFP (Roche; 5 μ l) followed by incubation with protein G-agarose (Santa Cruz, sc-2002; 5 μ l). After washing, CaNpl3-GFP was eluted by boiling in Laemmli buffer and analyzed by immunoblotting with anti-GFP and anti-ADMA (ASYM24; Upstate) antibodies.

PrA pull-down assays were performed essentially as described previously (26). Briefly, mid-log-phase cells expressing PrA fusion proteins were lysed in the lysis buffer described above with either 1% (Npl3) or 0.1% (Hmt1) Triton X-100. PrA fusion proteins and interacting proteins were precipitated with immunoglobulin G (IgG)-Sepharose (Pharmacia), washed, eluted with 3 M MgCl₂, precipitated with trichloroacetic acid, and analyzed by immunoblotting.

For immunoblot analyses, antibodies were used at the following dilutions: polyclonal anti-ScNpl3, 1:5,000 (7); monoclonal 1E4 (anti-methyl-ScNpl3) (38, 46), 1:2,500; polyclonal anti-ScHmt1 (27), 1:2,000; polyclonal anti-myc, 1:2,000 (sc-789; Santa Cruz Biotechnology); monoclonal anti-GFP (Roche), 1:1,000; and secondary anti-mouse-horseradish peroxidase and anti-rabbit-horseradish peroxidase, 1:5,000 (Amersham). Polyclonal anti-Hmt1 and anti-myc antisera also detect PrA fusion proteins. All proteins were visualized by enhanced chemiluminescence (Amersham), and migration was compared to that of Benchmark standards (Invitrogen).

In vivo labeling with S-adenosyl-[methyl-³H]-L-methionine (³H-AdoMet). *C. albicans* strains were grown at 30°C in YPD medium as previously described by Porras-Yakushi et al. (33), with the following changes. The strains were harvested by centrifugation at 1,000 \times g for 3 min at 4°C, and the pellets were washed a total of three times by the addition of 1 ml of sterile water followed by centrifugation for 5 min at 10,600 \times g at room temperature.

Fractionation by sodium dodecyl sulfate (SDS) gel electrophoresis was carried out as previously described (33). For fluorography, the gels were treated with

En³Hance (Perkin-Elmer Life Sciences) for 1 h and then washed for 20 min in water. Gels were dried in vacuo for a total of 3 h (2 h at 70°C and 1 h at room temperature).

Amino acid analysis. Analysis was performed as previously described by Niewmierzycka and Clarke (29) with the following modifications. After precipitation with trichloroacetic acid, lysates were centrifuged at 1,000 \times g for 20 min at 25°C. The pellets were washed once with 50 μ l of cold acetone, centrifuged at 1,000 \times g for 20 min at 25°C, and allowed to dry prior to being acid hydrolyzed. Hydrolysates were mixed with nonradiolabeled standards (50 μ l of 2 mM SDMA and 5 μ l each of 0.2 M ADMA and 0.2 M ω -MMA [Sigma Chemical Co., St. Louis, MO]) prior to being loaded on a Beckman AA-15 sulfonated polystyrene cation-exchange column (29).

Fluorescence microscopy. Cells expressing GFP-tagged CaNpl3 proteins were grown to mid-log phase in synthetic dropout medium lacking uridine, washed with phosphate-buffered saline (PBS), and incubated with 10 μ g/ml DAPI (4',6'-diamidino-2-phenylindole) in PBS for 30 min to visualize nuclei. Cells were washed with PBS and visualized by fluorescence microscopy (Olympus BX51; GFP and DAPI filters). Images were captured with an Evolution VF color digital camera (noncooled, 12-bit; MediaCybernetics) and QCapture Pro 5.0 software. For each filter, exposure times were equivalent for all strains.

RESULTS

Functional conservation of HMT1. A BLAST search of non-redundant databases with the major type I arginine methyltransferase from *S. cerevisiae* reveals a clear *C. albicans* ortholog. These proteins not only are very similar among numerous fungal species (Fig. 1A and data not shown), but also share a high degree of similarity with the predominant human PRMT, PRMT1 (Fig. 1A) (35, 41). To test whether PRMT function is conserved between these two fungal species, *C. albicans* *HMT1* (Ca*HMT1*) was subcloned into a plasmid under the control of the endogenous *S. cerevisiae* *HMT1* (*ScHMT1*) promoter (official gene names consist of three letters and a number, and therefore the Ca and Sc prefixes are used to distinguish orthologous genes from the two organisms). Although *HMT1* is not an essential gene in *S. cerevisiae*, Hmt1 is required in a strain that contains a point mutation in one of its substrates, the major mRNA-binding protein Npl3 (*npl3-1*) (24), or that lacks the 80-kDa mRNA cap-binding protein Cbp80 (*cbp80* Δ) (37). Plasmids that express *S. cerevisiae*, *C. albicans*, and human PRMTs were transformed into these *S. cerevisiae* strains. Cells expressing CaHmt1 grow as robustly as cells expressing ScHmt1 (Fig. 1B). Thus, *C. albicans* Hmt1 supports all essential Hmt1 functions in these *S. cerevisiae* strains. In contrast, expression of human PRMT1 allowed partial growth in the absence of Cbp80 but did not support growth of the *npl3-1* strain (Fig. 1B).

To test methyltransferase activity, these PRMT expression plasmids were transformed into *S. cerevisiae* cells lacking endogenous *HMT1* (*hmt1* Δ), and levels of Hmt1, Npl3, and methylated Npl3 were determined by immunoblotting (Fig. 1C). The antiserum raised against ScHmt1 (27) also recognizes CaHmt1, which migrates slightly faster, but not human PRMT1 (Fig. 1C, top panel); relative expression levels of the three PRMTs cannot be determined due to epitope differences, but all three PRMTs are expressed from the *ScHMT1* promoter. Although Npl3 levels are similar in all strains (Fig. 1C, middle panel), levels of methylated Npl3 vary depending on which methyltransferase is expressed. CaHmt1 methylates *S. cerevisiae* Npl3 almost as well as ScHmt1, whereas Npl3 methylation by the human enzyme is significantly reduced in comparison (Fig. 1C, bottom panel). Thus, *C. albicans* Hmt1

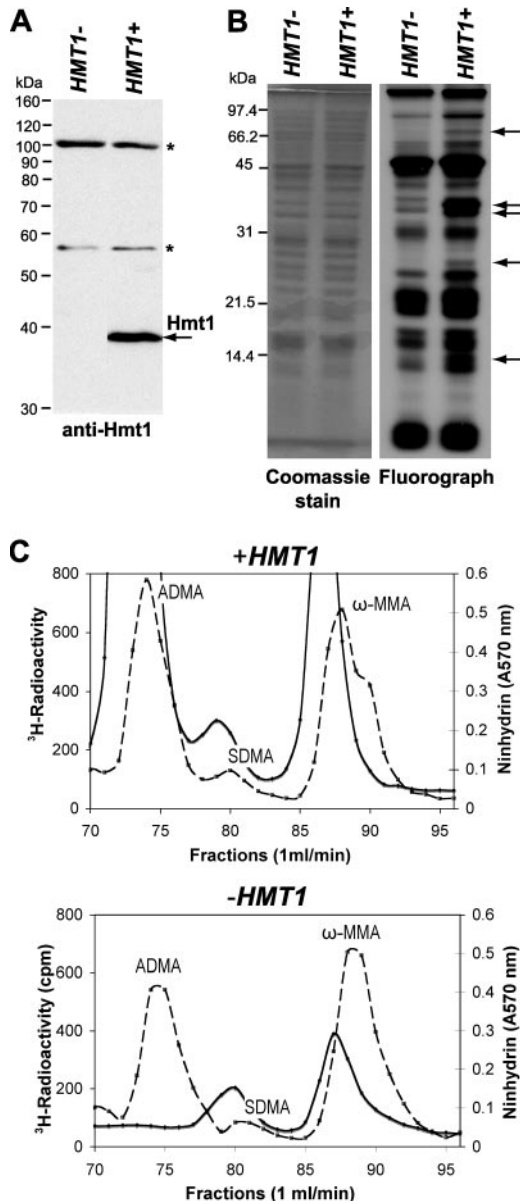


FIG. 2. *C. albicans* Hmt1 is not essential and is the major PRMT. (A) *hmt1Δ/hmt1Δ* (AMC11; *HMT1*⁻) and *hmt1Δ/hmt1Δ+HMT1* (AMC14; *HMT1*⁺) *C. albicans* strains were created using a PCR-based strategy. Total protein (10 μg) from these strains was analyzed by immunoblotting with anti-ScHmt1 antiserum. (B) *C. albicans* strains were labeled in vivo with [³H]AdoMet as described in Materials and Methods. Proteins in cell extracts (50 μg) were resolved by SDS gel electrophoresis and analyzed by Coomassie blue staining and fluorography. The dried gel was exposed to Kodak X-Omat AR film at -80°C for 10 days to detect methylated proteins. Arrows indicate major ³H-methylated polypeptides found in the *hmt1Δ/hmt1Δ+HMT1* strain (AMC14; *HMT1*⁺) expressing the methyltransferase and absent or greatly reduced in the mutant *hmt1Δ/hmt1Δ* strain (AMC11; *HMT1*⁻). (C) Amino acid analysis of *C. albicans* proteins from lysed cells in vivo labeled with [³H]AdoMet. Acid-hydrolyzed proteins (150 μg) were fractionated on a cation-exchange column as described in Materials and Methods. Radioactivity (³H) (solid line) was determined by scintillation counting. Nonradiolabeled amino acid standards of ADMA, ω-MMA, and SDMA were added to the hydrolysate and detected using a ninhydrin assay (dashed line). The upper panel shows the labeling in cells expressing Hmt1 (AMC14; *HMT1*⁺), and the lower panel shows the labeling in cells lacking Hmt1 (AMC11; *HMT1*⁻).

were created by homologous recombination. The entire *HMT1* gene was removed in *hmt1Δ/hmt1Δ* strains, and over 89% of *RMT2* was removed in *rmt2Δ/rmt2Δ* strains, including all methionine codons and the entire AdoMet-binding motif. Immunoblotting with the antibody raised against ScHmt1 revealed the presence of a ~39-kDa protein in the reconstituted strain that was absent in *hmt1Δ/hmt1Δ* cells (Fig. 2A). All *hmt1Δ/hmt1Δ* and *rmt2Δ/rmt2Δ* strains grew as robustly as reconstituted or heterozygous strains in rich media. The generation times of two *hmt1Δ/hmt1Δ* strains were 1.35 ± 0.08 h and 1.36 ± 0.02 h, compared to 1.36 ± 0.07 h and 1.32 ± 0.05 h for two reconstituted *hmt1Δ/hmt1Δ+HMT1* strains. Generation times of two *rmt2Δ/rmt2Δ* strains were 1.37 ± 0.09 h and 1.34 ± 0.06 h, compared to 1.32 ± 0.07 h for the *rmt2Δ/RMT2* heterozygous strain. Therefore, similar to the case for the *S. cerevisiae* orthologs (18, 24, 29), neither *HMT1* nor *RMT2* is essential for *C. albicans* growth.

To test PRMT activity in vivo, *C. albicans* cells with and without each methyltransferase were grown in the presence of ³H-AdoMet. Radiolabeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography and acid hydrolyzed for amino acid analysis to identify methylarginine species. Radioactive labeling of multiple proteins was lower in lysates of *hmt1Δ/hmt1Δ* cells than in those of *hmt1Δ/hmt1Δ+HMT1* cells (Fig. 2B, right panel), suggesting lower levels of arginine methylation of these substrates in the absence of Hmt1. The radioactive ADMA peak visible in acid hydrolysates of proteins from the *hmt1Δ/hmt1Δ+HMT1* strain (Fig. 2C, upper panel) is absent in *hmt1Δ/hmt1Δ* acid hydrolysates; the ω-MMA peak is also significantly reduced in *hmt1Δ/hmt1Δ* cells (Fig. 2C, lower panel). These results indicate that, as in *S. cerevisiae* (18), Hmt1 is the major type I arginine methyltransferase in *C. albicans*.

In contrast, levels of radioactively labeled proteins are similar in *rmt2Δ/RMT2* and *rmt2Δ/rmt2Δ* cells (Fig. 3A, right panel). This result is consistent with the presence of only one known Rmt2 substrate in *S. cerevisiae*: Rmt2 monomethylates ribosomal protein L12 (Rpl12) in *S. cerevisiae* at a single site (10), and CaRpl12 may comigrate with strongly labeled proteins between the 14.4- and 21.5-kDa markers. In amino acid analysis chromatography, δ-MMA migrates similarly to SDMA (29), and, as predicted, a small peak that elutes close to the SDMA standard in the *rmt2Δ/RMT2* and *HMT1* samples is reduced in *rmt2Δ/rmt2Δ* hydrolysates (Fig. 3B). These results are consistent with Rmt2 catalyzing δ-MMA formation in *C. albicans*. The presence of a small amount of radioactivity in the δ-MMA position suggests that an additional methyltransferase activity may be present that forms symmetric dimethylarginine residues, although no gene that may encode this activity has been identified.

Identification of a *C. albicans* Npl3 ortholog. The first cellular function demonstrated for arginine methylation in vivo was a role in nuclear export of two Hmt1 substrates in *S. cerevisiae*,

The radioactivity in the off-scale fractions of the upper panel is 2,625 for fraction 72, 5,707 for fraction 73, and 1,172 for fraction 87. In these experiments, the ³H-labeled methylated derivatives eluted slightly earlier than the nonisotopically labeled standards due to an isotope effect (18).

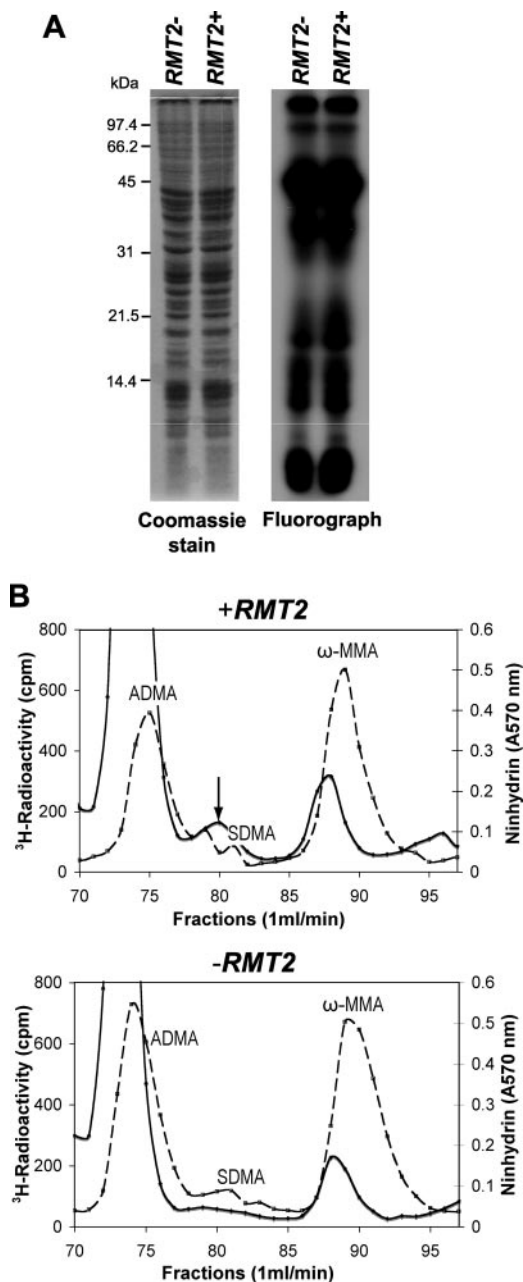


FIG. 3. *C. albicans* Rmt2 catalyzes the formation of δ -MMA as a minor PRMT. (A) *C. albicans* strains *RMT2/rmt2 Δ* (AMC28; *RMT2*⁺) and *rmt2 Δ /rmt2 Δ* (AMC30; *RMT2*⁻) were labeled in vivo with [³H]AdoMet as described in Materials and Methods. Proteins in cell extracts (50 μ g) were analyzed by SDS gel electrophoresis as described in the legend to Fig. 2. The dried gel was exposed to Kodak BioMax MS film at -80°C for 10 days. (B) Amino acid analysis of *C. albicans* proteins from lysed *RMT2/rmt2 Δ* and *rmt2 Δ /rmt2 Δ* cells labeled in vivo with [³H]AdoMet as described in the legend Fig. 2. The upper panel shows the labeling in cells expressing Rmt2 (AMC28; *RMT2*⁺), and the lower panel shows the labeling in *rmt2 Δ /rmt2 Δ* cells (AMC30; *RMT2*⁻). The arrow indicates the peak that decreases in the absence of Rmt2. The radioactivity in the off-scale fractions for the upper panel is 2,062 for fraction 73, 2,759 for fraction 74, and 1,079 for fraction 75; for the lower panel it is 1,973 for fraction 73 and 1,411 for fraction 74. The radioactive derivatives are expected to elute slightly earlier than the standards, as described in the Fig. 2 legend.

including the mRNA-binding protein Npl3 (37). Subsequent studies demonstrated that methylation facilitates nuclear import of human RNA helicase A (40) and that PRMT1 deletion increased cytoplasmic localization of mammalian RNA-binding protein Sam68 (12), also suggesting a role for methylation in import. We therefore wished to test whether arginine methylation has a role in either nuclear export or import of methylated RNA-binding proteins in *C. albicans*. A putative Npl3 ortholog was identified in the *C. albicans* genome (Fig. 4A). Npl3 is not as highly conserved between fungal species as is Hmt1, but *S. cerevisiae* Npl3 and its *C. albicans* ortholog both contain two RNA recognition motifs and a C-terminal domain with many arginine-glycine-glycine (RGG) tripeptides that are extensively methylated by Hmt1 in *S. cerevisiae* (26). The two proteins also share a heptapeptide at the C terminus that includes a serine targeted by the SR protein kinase in *S. cerevisiae*, Sky1 (20, 50).

To test whether Npl3 was functionally conserved between fungal species, we subcloned full-length *C. albicans* *NPL3* (*CaNPL3*) into an *S. cerevisiae* PrA expression vector. A second plasmid expresses a PrA-tagged chimeric protein containing the first three domains of ScNpl3, the RGG domain of CaNpl3, and the last 11 amino acids of ScNpl3. These plasmids and control plasmids were transformed into *npl3 Δ* *S. cerevisiae* cells bearing a *URA3* plasmid for ScNpl3 expression. After growth on 5-FOA medium, cells expressing the chimeric protein grew as well as those expressing ScNpl3, but cells expressing full-length CaNpl3 grew only slightly better than cells bearing the expression vector (Fig. 4B). Therefore, although all proteins are expressed at similar levels (Fig. 4C), the RGG domain of CaNpl3 can replace essential functions of the ScNpl3 RGG domain, but the full-length *C. albicans* protein cannot perform functions essential for growth of the *npl3 Δ* cells.

Methylation of ScNpl3 disrupts protein-protein interactions, including Npl3 self-association (49). The RGG domain helps direct ScNpl3 self-association, since PrA-ScNpl3 can interact with the RGG domain alone in *hmt1 Δ* cells (A. McBride, unpublished data). Therefore, we tested whether the CaNpl3 RGG domain could mediate methylation-sensitive Npl3-Npl3 interaction in *S. cerevisiae* (Fig. 4C). The PrA-Npl3 plasmids were transformed into Δ *hmt1* and *HMT1* cells expressing myc-tagged ScNpl3 and PrA-Npl3, and associated proteins were isolated with IgG-Sepharose. Whereas the chimeric Npl3 showed slightly less interaction than ScNpl3 with ScNpl3-myc, binding of full-length CaNpl3 to ScNpl3-myc was severely reduced, suggesting that domains other than the RGG domain influence ScNpl3 self-association. In all cases, however, Npl3-Npl3 binding is decreased in the presence of *HMT1* (Fig. 4C).

Methylation and nucleocytoplasmic transport of Npl3 in *C. albicans*. The methylation sensitivity of Npl3 dimerization in *S. cerevisiae* has been linked to the role of arginine methylation in ScNpl3 export (26). Given the increased interaction of Npl3 with proteins containing the CaNpl3 RGG domain in *hmt1 Δ* *S. cerevisiae* cells, we tested whether Npl3 is methylated in *C. albicans* and whether Hmt1 influences its nuclear transport. To detect Npl3 methylation in *C. albicans*, a GFP tag was inserted at the C terminus of one of the endogenous *NPL3* alleles. Npl3-GFP immunoprecipitated from cells with or without Hmt1 was detected with anti-GFP antibodies to detect relative protein levels and with an antidimethylarginine antiserum to

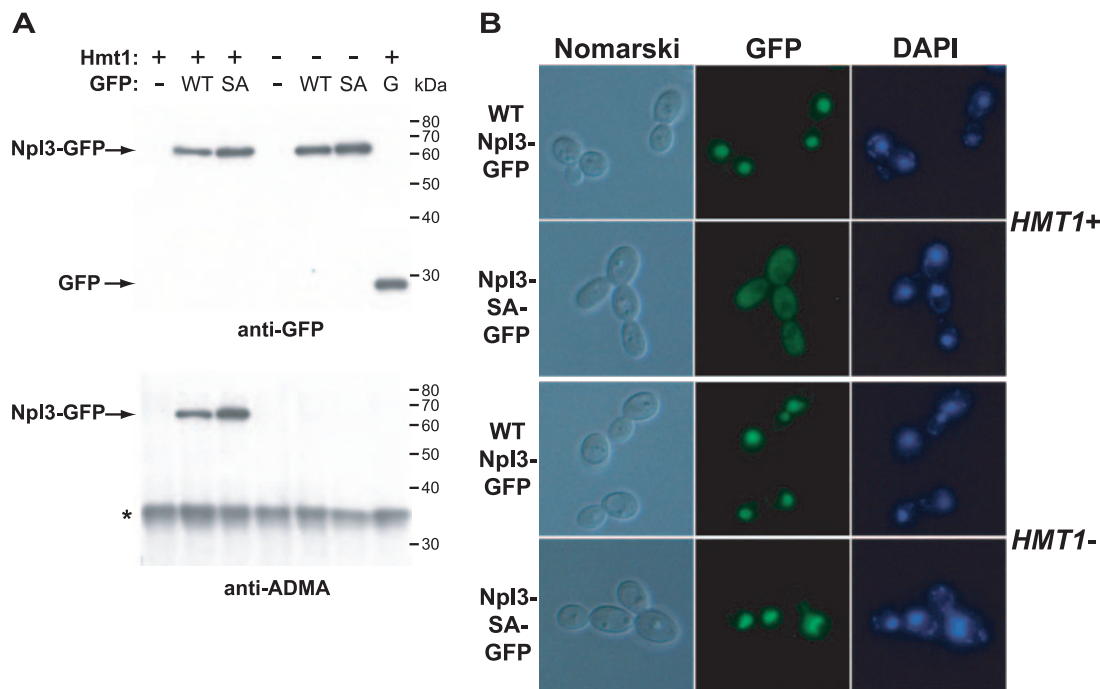


FIG. 5. Hmt1 influences nucleocytoplasmic transport of *C. albicans* Npl3. (A) *NPL3* was GFP tagged by homologous recombination in homozygous *C. albicans* strains with (+; AMC46 and AMC47) or without (-; AMC48 and AMC49) Hmt1. PCR products used for recombination contained either wild-type *NPL3* sequences (WT) or introduced a T-to-G mutation in codon 340, encoding an S-to-A (SA) mutation in the C terminus of Npl3. Equal amounts of total protein from mid-log-phase GFP-tagged and parental cells were immunoprecipitated with anti-GFP and PrG-Sepharose. Equal amounts of each precipitate were analyzed by immunoblotting with anti-GFP (from 0.3 mg total protein) and antimethylarginine antiserum (from 0.6 mg total protein). Parental strains lacking GFP (-; BWP17 [+Hmt1] and AMC36 [-Hmt1]) and an *HMT1/HMT1* strain expressing GFP alone (G; MLR62) were used as controls. (B) *HMT1/HMT1* (*HMT1*+) and *HMT1*- (*HMT1*-; AMC48 and AMC49) strains were grown to mid-log phase and stained with DAPI. Cells were visualized by Nomarski and fluorescence microscopy using GFP and DAPI filters. For each filter, exposure times were equivalent for all strains.

DISCUSSION

Sequencing of the *C. albicans* genome has revealed an intriguing complement of PRMT genes. This species contains a clear ortholog of the ubiquitous type I methyltransferase Hmt1 as well as a type IV arginine methyltransferase, Rmt2, but it lacks a clear type II Hsl7/PRMT5 ortholog. Given the presence of type II enzymes in many other eukaryotes and the apparent specificity of type IV enzymes to fungal and plant species, *C. albicans* offers a particularly interesting system in which to explore roles of these eukaryotic enzymes.

Although type I PRMT sequences are highly conserved among eukaryotes, the *in vivo* comparison of *S. cerevisiae*, *C. albicans*, and human type I PRMTs demonstrates functional differences that correlate with sequence similarity (Fig. 1). CaHmt1 supports growth of *S. cerevisiae* strains that require Hmt1, whereas human PRMT1 supports partial growth of a *cbp80Δ* strain but does not allow growth of an *npl3-1* strain. In these experiments all PRMTs were expressed from the ScHmt1 promoter to normalize transcription levels. Therefore, although PRMT1 protein levels are not known, this result agrees with the finding that overexpression of this PRMT1 isoform is required for growth of an *hmt1Δ npl3-1* strain (35). Similarly, the two fungal PRMTs methylated Npl3 to a greater extent than did human PRMT1. The ability of fungal Hmt1 proteins to heterodimerize, combined with the similarity in their antenna sequences and in the region of the AdoMet-

binding domain sequences to which the antenna binds, suggests that CaHmt1 is also likely to dimerize *in vivo*.

Amino acid analysis of radiolabeled proteins in *hmt1Δ/hmt1Δ* and *rmt2Δ/rmt2Δ* *C. albicans* cells reveals that both Hmt1 and Rmt2 function as arginine methyltransferases in this fungus. The radiolabeling of a number of proteins decreases in *hmt1Δ/hmt1Δ* cells, and the decrease in the ADMA and MMA peaks confirms that CaHmt1 is a type I methyltransferase. The residual labeled proteins in *hmt1Δ/hmt1Δ* cells may indicate other types of protein methylation, such as lysine methylation, or potentially conversion of tritiated AdoMet to methionine and translational incorporation. No decrease is seen in overall protein methylation in *rmt2Δ/rmt2Δ* cells; this result is consistent with only one Rmt2 substrate, ribosomal protein L12, being identified in *S. cerevisiae* (10). The decrease in a peak that migrates close to the SDMA standard, however, supports the identification of CaRmt2 as a type IV methyltransferase.

Interestingly, enlargement of the region around the SDMA standard in the *rmt2Δ/rmt2Δ* sample reveals a small residual peak (Fig. 3B). No such peak was seen in *rmt2Δ* *S. cerevisiae* hydrolysates (29). In addition, there is a small residual peak in the ω -MMA region of the *hmt1Δ/hmt1Δ* sample (Fig. 2C). *C. albicans* may, therefore, express one or more PRMTs that are not identified through BLAST searches; such an enzyme might be a type II PRMT, which would catalyze SDMA and ω -MMA region formation. The anti-ScHmt1 antiserum recognizes two

proteins larger than CaHmt1 in *hmt1Δ/hmt1Δ* cells (Fig. 2A). Although it is intriguing to consider whether one of these proteins might be another *C. albicans* PRMT, neither represents Rmt2, since both proteins are detected in *rmt2Δ/rmt2Δ* lysates (A. McBride, unpublished data). Human PRMT1 is more similar to fungal Hmt1 proteins than to the human type II enzyme PRMT5 (17), yet PRMT1 is not recognized by the antiserum (Fig. 1b). Therefore, neither of the higher-molecular-weight proteins recognized by the anti-ScHmt1 antiserum is likely to be a type II PRMT. The putative enzyme or enzymes responsible for residual peaks in *C. albicans hmt1Δ/hmt1Δ* and *rmt2Δ/rmt2Δ* cells therefore remain to be identified.

To address the *in vivo* function of *C. albicans* Hmt1, we developed an assay to monitor nuclear export of the putative *C. albicans* Npl3 ortholog, a likely Hmt1 substrate. In *S. cerevisiae*, Npl3 shuttles between the nucleus and the cytoplasm but is predominantly nuclear at steady state (7, 16). Sky1 phosphorylation of ScNpl3 influences its binding to mRNA and import factor Mtr10, and deletion of Sky1 or mutation of its target site in ScNpl3 slows Npl3 nuclear import (20, 50). In our assay, an S-to-A point mutation at the putative phosphorylation site was used to slow import of CaNpl3-GFP, allowing detection of Npl3 export defects in *hmt1Δ/hmt1Δ* cells. Unlike the *S. cerevisiae* nuclear export assay (25), this assay detects steady-state localization and does not allow repression of Npl3-GFP synthesis before import is inhibited; therefore, we cannot rule out that Hmt1 may possibly slow Npl3 import rather than facilitate export. Regardless of the mechanism, methylation of CaNpl3 clearly favors export of this shuttling RNA-binding protein, in contrast to the role of methylation in expediting import of mammalian RNA-binding proteins RNA helicase A and Sam68 (12, 40).

The presence of eight RS/SR dipeptides in the C terminus of *S. cerevisiae* Npl3, including six within the RGG domain, has led to comparisons with mammalian SR proteins (38, 50), particularly because the yeast SR protein kinase Sky1 targets the final RS dipeptide. Only this final RS dipeptide, which is found in the context of a conserved heptapeptide, is found in the *C. albicans* Npl3 protein (Fig. 4A). Five other fungal species also encode Npl3-like proteins with RNA recognition motifs, RGG domains, and similar C-terminal heptapeptides with the consensus sequence R(E/D)RSP(T/V)R (*Candida glabrata*, ORF CAGL0H04763g; *Debaryomyces hansenii*, ORF DEHA0D05115g; and *Kluyveromyces lactis*, ORF KLLA0B00979g [14] and *Ashbya gossypii*, ORF ADR183Cp [13]). Within the RGG domains of these five proteins, only a single SR is found, in the RGG domain of the *Ashbya gossypii* Npl3-like protein. These comparisons, combined with the ability of the chimeric ScNpl3 protein bearing the CaNpl3 RGG domain to support wild-type growth of *S. cerevisiae* lacking Npl3, suggest that this family of likely RNA-binding proteins does not require RS/SR dipeptides within the RGG domain for function.

The *C. albicans* genome encodes a number of likely Hmt1 substrates in addition to Npl3. Although some of these proteins with RG-rich domains are orthologous to known targets for methylation in *S. cerevisiae*, other RGG-containing proteins in one species share sequences with proteins that lack RGG domains in the other fungus. The *hmt1Δ/hmt1Δ C. albicans* strains described in this study will allow the exploration of these differences, including testing whether arginine methyl-

ation affects the function of these proteins. In addition, the presence of residual methylarginine peaks in protein hydrolysates of *hmt1Δ/hmt1Δ* and *rmt2Δ/rmt2Δ C. albicans* suggests that future work may uncover another PRMT in this pathogenic fungus.

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ADDENDUM IN PROOF

The Npl3 protein as defined in assembly 20 of the *C. albicans* sequencing project (Fig. 4A) contains 58 more N-terminal amino acids than the assembly 19 Npl3 used in the experiments shown in Fig. 4B and C.

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