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* npl3Δ 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 rmt2Δ/rmt2Δ 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, Hs7, 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-N⁶-monomethylarginine (ω-MMA) and asymmetric N⁶,N³-di-methylarginine (ADMA) (18). Hs7 catalyzes formation of ω-MMA (28) and shows sequence similarity to mammalian PRMT5, which forms ω-MMA and symmetric N⁶,N³-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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**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 (14). For *S. cerevisiae* strains,YPD and SD media were supplemented with 80 μg/ml uridine. Generation times for *H. sapiens* PRMT1 and *C. albicans* HMT1 were determined by diluting overnight cultures to an optical density at 650 nm. Generation times for strains were determined by diluting overnight cultures to an optical density at 600 nm (OD<sub>600</sub>) of 0.1 in YPD and monitoring the OD<sub>600</sub> every 30 min. For each strain, the log of normalized OD<sub>600</sub> values was graphed as a function of time, and the linear part of the graph was used to calculate generation times.

### Table 1. Strains used in this study

| Species and strain | Genotype                  | Source or reference |
|-------------------|---------------------------|---------------------|
| *S. cerevisiae*    |                           |                     |
| FY23              | MATα ura3·52 trp1Δ63 leu2Δ1 GAL+ | F. Winston          |
| PSY865            | MATα hmt1Δ:HIS3 ade2 ade8 ura3 leu2 his3 yl1 | 24                  |
| PSY866            | MATα ade8 ade8 ura3 leu2 his3 yl1 Δhmt1:HIS3 npl3·1 + pPS1307 | 24                  |
| PSY814            | MATα npl3Δ:HIS3 ade2 ade8 can1 ura3 leu2 his3 yl1 trp1 + YCp50-NPL3-3 | 23                  |
| PSY1191           | MATα ade8 ade8 ura3 leu2 his3 Δhmt1:HIS3 Δhbs80:HIS3 + pPS1307 | 37                  |
| YAM533            | MATα hmt1Δ:HIS3 NPL3-myc::URA3 ade2 ade8 ura3 leu2 his3 yl1 | 26                  |
| YAM535            | MATα NPL3-myc::URA3 ade2 ade8 ura3 leu2 his3 yl1 | 26                  |
| *C. albicans*     |                           |                     |
| AMC11             | hmt1Δ:HIS1 ura3Δ::imm344::URA3-IRO1 arg4::hisG his1::hisG | This study          |
| AMC14             | hmt1Δ::ARG4 ura3Δ::imm344 arg4::hisG his1::hisG | This study          |
| AMC28             | mt2Δ::HIS1 ura3Δ::imm344::URA3-IRO1 arg4::hisG his1::hisG | This study          |
| AMC30             | mt2Δ::ARG4 ura3Δ::imm344 arg4::hisG his1::hisG | This study          |
| AMC35             | hmt1Δ::ARG4 ura3Δ::imm344 arg4::hisG his1::hisG | This study          |
| AMC36             | hmt1Δ::ARG4 ura3Δ::imm344 arg4::hisG his1::hisG | This study          |
| AMC46             | NPL3-GFP::URA3 ura3Δ::imm344 arg4::hisG his1::hisG | This study          |
| AMC47             | NPL3-SA-GFP::URA3 ura3Δ::imm344 arg4::hisG his1::hisG | This study          |
| AMC48             | NPL3-GFP::URA3 hmt1Δ::HIS1 ura3Δ::imm344 arg4::hisG his1::hisG | This study          |
| AMC49             | NPL3-SA-GFP::URA3 hmt1Δ::HIS1 ura3Δ::imm344 arg4::hisG his1::hisG | This study          |
| BW17              | npl3Δ::imm344 arg4::hisG his1::hisG | 45                  |
| MLR62             | ura3Δ::imm344 ARG4::URA3 arg4::hisG his1::hisG::pTEF1-GFP | A. Mitchell         |

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 from *C. albicans* 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 Ndel and NolI and inserted into Ndel-NolI-digested pPS13872 to produce pAM160 (CaHMT1) and pAM161 (HsPRMT1), which express arginine methyltransferases with an additional MH diepoxide from *S. cerevisiae* Hmt1 at the C terminus. The protein A (PrA)-SCHMT1 expression plasmid pAM91 was constructed by inserting an Ndel-Msel fragment from pPS1872 into Ndel-Smal-digested pNPPATA. The URA3 CaHMT1 plasmid pAM390 was constructed by inserting an Xbal-HindIII fragment from pAM160 into Xbal-HindIII-digested pRS316. The *C. albicans* HMT1 gene and surrounding regions were cloned by PCR amplification of BW17 genomic DNA with oligonucleotides AM113 and AM114, Xhol and Spel digestion, and insertion into Xhol-Spel-digested pRS315, resulting in pAM322. The *C. albicans* HMT1 reconstitution plasmid (pAM385) resulted from the insertion of an Xhol-BerBI fragment of pAM322 into Xhol-Smal-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 BW17 genomic DNA with oligonucleotides AM143 and AM144, digested with Ndel and BamHI, and inserted into Ndel-BamHI-digested pNPPATA to create the PrA-CaNPL3 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 Apal site was introduced into the PrA-SaNPL3 plasmid pPS2389 through a silent G-to-C mutation in the Gly312 codon. The *C. albicans* NPL3 regulatory regions were cloned by PCR amplification of BW17 genomic DNA with 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 Apal-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...
The University of Maine, Orono, DNA sequencing facility to verify proper fusion of the original reference strain to fill in gaps (1), these 111 nucleotides are identical in our genome sequence at the time of strain construction.

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 hmt1 gene at its endogenous locus by transformation of PstI-NotI-digested pBSK-ARG4 (from pRSARG4/H9004/hmt1/36 to the HMT1 ORF; pAM385 and selection on medium lacking uridine. The hmt1 marker was tagged with the green fluorescent protein (GFP) gene in NPL3, deleted restriction site.

### Table 2. Oligonucleotides used in this study

| Oligonucleotide | Sequence (5'→3'*) | Site/mutationa |
|-----------------|-------------------|---------------|
| AM173 AM154 | GGATACGGTGATGTATTTT | NeoL– |
| AM147 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
| AM173 AM170 | GTGATATGATAGAGGTGGATACGATCGTGATACTTCAACGACAGGAGGGATGATCATACGATCGTGAAAGAgCTCCAACCCGTTTTggtggtggttctaaaggtgaagaattatt | NeoL– |
**TABLE 3. Plasmids used in this study**

| Plasmid       | Features             | Source or reference |
|---------------|----------------------|---------------------|
| pAM91         | CEN LEU pNop-PrA-SchMT1 Amp′ | This study          |
| pAM160        | CEN LEU2 SchMT1p-CaHMT1 Amp′ | This study          |
| pAM161        | CEN LEU2 SchMT1p-HsPrMT1 Amp′ | This study          |
| pAM322        | CEN LEU2 CaHMT1 Amp′ | This study          |
| pAM361        | CEN LEU pNop-PrA-CaNPL3 Amp′ | This study          |
| pAM362        | CEN LEU pNop-PrA-CaNPL3-CaRGG Amp′ | This study          |
| pAM385        | URA3 CaHMT1 Amp′ | This study          |
| pAM390        | CEN URA3 CaHMT1 Amp′ | This study          |
| pAM463        | CEN LEU pNop-PrA-CaNPL3 Apa1 Amp′ | This study          |
| pBSK-URA      | URA3 Amp′ | 30                  |
| pDS10         | URA3 Amp′ | 30                  |
| pGEMHIS1      | HIS1 Amp′ | 45                  |
| pMG1602       | GFP URA3 Amp′ | 19                  |
| pNOPPATA      | CEN LEU pNop-PrA Amp′ vector | 22                  |
| pPS1302       | GST-HsPrMT1v2 Amp′ | 35                  |
| pPS1307       | CEN URA3 SchMT1 Amp′ | 24                  |
| pPS1872       | CEN LEU2 SchMT1 Amp′ | 27                  |
| pPS2389       | CEN LEU pNop-PrA-CaNPL3 Amp′ | 49                  |
| pPS2575       | CEN URA3 SchMT1-Δmonenn Amp′ | 44                  |
| pRSARG4ΔSpeI  | ARG4 Amp′ | 45                  |
| pRS315        | CEN LEU2 Amp′ vector | 39                  |
| pRS316        | CEN URA3 Amp′ 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 μl) followed by incubation with protein G-agarose (Santa Cruz, sc-2002; 5 μl). 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 pulldown 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-Npl3, 1:5,000 (7); monoclonal 1E4 (anti-methyl-ScNpl3) (38, 46), 1:2,500; polyclonal anti-ScHmt1, 1:2,000 (sc-789; Santa Cruz Biotechnology); monoclonal anti-GFP (Roche), 1:1,000; and 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 (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 for immunoblotting. Amino acid analysis. Analysis was performed as previously described by Niew-merzycka and Clarke (29) with the following modifications. After precipitation with trichloroacetic acid, lysates were centrifuged at 1,000 × g for 20 min at 25°C. The pellets were washed once with 50 μl of cold acetone, centrifuged at 1,000 × g for 20 min at 25°C, and allowed to dry prior to being acid hydrolyzed. Hydrolysates were mixed with nonradioabeled standards (50 μl of 2 mM SDMA and 5 μl 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 μg/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 EvolutionVF 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* (CaHMT1) was subcloned into a plasmid under the control of the endogenous *S. cerevisiae* HMT1 (SchHMT1) 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 (np1-3-1) (44), or that lacks the 80-kDa mRNA cap-binding protein Cbp80 (cbp80Δ) (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 SchHmt1 (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 np1-3-1 strain (Fig. 1B).

To test methyltransferase activity, these PRMT expression plasmids were transformed into *S. cerevisiae* cells lacking endogenous HMT1 (hmt1Δ), and levels of Hmt1, Npl3, and methylated Npl3 were determined by immunoblotting (Fig. 1C). The antiserum raised against SchHmt1 (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 SchHMT1 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 SchHmt1, whereas Npl3 methylation by the human enzyme is significantly reduced in comparison (Fig. 1C, bottom panel). Thus, *C. albicans* Hmt1

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**EnHance (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).**
demonstrates significant methyltransferase activity for a known Hmt1 substrate in *S. cerevisiae*. The crystal structures of *S. cerevisiae* Hmt1 and human PRMT1 reveal PRMT dimerization, which is important for enzyme function (44, 51). To determine whether *C. albicans* Hmt1 showed similar protein-protein interactions, heterodimerization of CaHmt1 and ScHmt1 proteins was tested. CaHmt1 was coexpressed with a PrA-tagged form of ScHmt1 in *hmt1Δ*/*H9004* *S. cerevisiae* cells. After PrA-ScHmt1 isolation, untagged Hmt1 proteins associated with PrA-ScHmt1 were detected by immunoblotting (Fig. 1D). Both ScHmt1 and CaHmt1 copurify with PrA-ScHmt1, supporting cross-species Hmt1 hetereodimerization (lane 3, top panel) as well as homodimerization of ScHmt1 (lane 2). The presence of a ~40-kDa copurifying protein depends on the presence of untagged Hmt1 (lanes 1 and 2), the presence of the Hmt1 dimerization domain (lanes 2 and 4) (44) and the expression of PrA-ScHmt1 (lanes 2 and 5). The lower band in the *C. albicans* lane is unlikely to represent a degradation product of PrA-ScHmt1, since the protein in this lane migrates slightly faster than the minor band seen in the absence of untagged Hmt1 expression (lane 1). The ability of *C. albicans* Hmt1 to bind to *S. cerevisiae* Hmt1, combined with the conservation of regions involved in ScHmt1 dimerization (Fig. 1A) suggests that the *C. albicans* enzyme may dimerize in vivo. Hmt1, the major PRMT in *C. albicans*. To determine whether *HMT1* and *RMT2* genes encode functional PRMTs in *C. albicans*, strains with homozygous deletions of each gene were examined.

FIG. 1. Hmt1 is functionally conserved between *S. cerevisiae* and *C. albicans*. (A) Clustal W alignment (9) of type I arginine methyltransferase proteins *Saccharomyces cerevisiae* Hmt1 (*S. cer.*), *Candida albicans* Hmt1 (*C. alb.*), and human PRMT1 (*H. sap.*). HRMT1L2v.1 (35). Indicated motifs include conserved methyltransferase motifs (I, post-I, II, and III) that mediate binding to the enzyme cofactor AdoMet, double-E (bold) and THW loop motifs common to protein methyltransferases, and the “antenna” domain (positions 175 to 204) that mediates dimerization of *S. cerevisiae* Hmt1 through hydrophobic interactions with the AdoMet-binding domain (dashed boxes) (44). Asterisks denote identical residues in all sequences, colons denote conserved substitutions, and periods denote semiconserved substitutions. (B) *S. cerevisiae* strains that require *HMT1* due to the presence of the *npl3-1* mutation (PSY866) or deletion of the 80-kDa cap-binding protein gene (PSY1191) were transformed with *CEN LEU2* plasmids that express either no Hmt1 (vector, pRS315) or one of the arginine methyltransferases shown in panel A (*Sc*, *S. cerevisiae* Hmt1, pPS1872; *Ca*, *C. albicans* Hmt1, pAM160; *Hs*, *H. sapiens* PRMT1, pAM161). The functionality of each methyltransferase was tested by incubation on medium containing 5-FOA, which selects for loss of a *URA3* *Sc HMT1* expression plasmid, at 25°C (*hmt1Δ npl3-1*) or 30°C (*hmt1Δ cbp80*) for 3 days. (C) An *hmt1Δ* *S. cerevisiae* strain (PSY865) was transformed with the plasmids described for panel B, and grown at 30°C to mid-log phase, and lysed in radioimmunoprecipitation assay buffer. Total protein (5 μg) was analyzed by immunoblotting with polyclonal antisera raised against ScHmt1 (27) and ScNpl3 (7) from *S. cerevisiae* and a monoclonal antibody that specifically recognizes methylated ScNpl3 (1E4) (38, 46). (D) A PrA fusion to *S. cerevisiae* Hmt1 (†) was expressed from pAM91 in cells expressing untagged ScHmt1 (Sc; pPS1307), CaHmt1 (Ca; pAM390), or ScHmt1 lacking the antenna domain (Sc m; pPS2575). CaHmt1 was also expressed in the presence of the PrA vector (−; pNOPPATA). Proteins from mid-log-phase cells were precipitated with IgG-Sepharose. Proteins isolated from 0.5 mg lysate (beads) and 10 μg lysates were analyzed by immunoblotting with anti-ScHmt1 antisera, which also binds to PrA.
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 met-thonine 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 hmt1Δ/hmt1Δ strain (Fig. 2A) 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.

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 nonsotopically labeled standards due to an isotope effect (18).
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 npl3Δ S. cerevisiae cells bearing a URA3 plasmid for Snp3 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 npl3Δ 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 hmt1Δ 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 Δhmt1 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 hmt1Δ 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...
determine relative methylation levels (Fig. 5A). The methylation-specific antiserum only detected Npl3-GFP proteins in cells expressing \textit{HMT1}. Immunoprecipitates from cells lacking the GFP tag or expressing the GFP tag alone do not contain any proteins recognized by the dimethylarginine antiserum. Therefore, Npl3 is an \textit{Hmt1} substrate in \textit{C. albicans}.

Fluorescence microscopy revealed that wild-type Npl3-GFP colocalizes with DAPI staining and is therefore nuclear in \textit{C. albicans} in the presence or absence of \textit{Hmt1} (Fig. 5B). Although this result indicates that \textit{Hmt1} is unlikely to have a role in expediting nuclear import, inhibitory effects of \textit{HMT1} deletion on nuclear export cannot be determined in these cells. In \textit{S. cerevisiae}, nuclear export defects have been probed using a temperature-sensitive nucleoporin mutation that slows Npl3 import (25, 37). The diplody of \textit{C. albicans} and paucity of selectable markers led us to devise another strategy to slow Npl3 nuclear import to test the effect of \textit{Hmt1} on Npl3 export in \textit{C. albicans}. An S-to-A mutation in the serine that is phosphorylated by Sky1 decreases Npl3 import in \textit{S. cerevisiae} (20, 50). The amino acids surrounding this serine in the C terminus of Npl3 are conserved in \textit{C. albicans} (Fig. 4A), which also contains orthologs of Sky1 and Mtr10, the Npl3 import receptor (2, 36). \textit{Hmt1} methylates npl3-S340A in \textit{C. albicans} (Fig. 5A), and npl3-S340A localizes throughout \textit{HMT1} cells at steady state (Fig. 5B), indicating that the rate of Npl3 import relative to export is decreased by this mutation. When this mutation was introduced into \textit{hmt1}/\textit{H9004} cells, however, npl3-SA–GFP showed nuclear localization similar to that of wild-type Npl3-GFP (Fig. 5b). This result demonstrates that \textit{Hmt1} deletion lowers the rate of npl3-SA–GFP export relative to that of import, suggesting that \textit{Hmt1} facilitates nuclear export of Npl3 in \textit{C. albicans}.

**FIG. 4.** The RGG domain of \textit{C. albicans} Npl3, but not full-length protein, can function in \textit{S. cerevisiae}. (A) Clustal W alignment (9) of \textit{S. cerevisiae} RNA-binding protein Npl3 (\textit{S. cer.}) and the \textit{C. albicans} Npl3 ortholog (\textit{C. alb.}). RNA recognition motifs (RRMs) and arginine-glycine-rich (RGG) domains are shown. Sky1-mediated phosphorylation of serine 411 (bold) in the conserved C-terminal heptapeptide facilitates Npl3 nuclear import in \textit{S. cerevisiae} (20, 50). SR dipeptides found in \textit{S. cerevisiae}, but not \textit{C. albicans}, are underlined. Asterisks denote identical residues in all sequences, colons denote conserved substitutions, and periods denote semiconserved substitutions. (B) An \textit{S. cerevisiae} strain lacking \textit{NPL3} (PSY814) was transformed with plasmids that express PrA alone (vector; \textit{pNOPPATA}) or PrA fused to \textit{S. cerevisiae} Npl3 (\textit{ScNpl3}; \textit{pPS2389}), \textit{C. albicans} Npl3 (\textit{CaNpl3}; \textit{pAM361}), or a chimeric protein composed of the first three domains of \textit{ScNpl3} with the RGG domain of \textit{CaNpl3} (\textit{ScNpl3-CaRGG}; \textit{pAM362}). Cells were grown overnight to mid-log phase in medium lacking leucine and washed, and 10-fold dilutions (10⁶ to 10³ cells) were plated on 5-FOA medium and grown at 30°C for 2 days. (C) \textit{hmt1Δ} (YAM533) and \textit{HMT1} (YAM535) \textit{S. cerevisiae} strains expressing myc-tagged ScNpl3 were transformed with the PrA-Npl3 plasmids shown in panel B. Proteins from mid-log-phase cells were precipitated with IgG-Sepharose. Proteins isolated from 1.7 mg lysate (beads) and total lysates (20 µg) were analyzed by immunoblotting with a polyclonal anti-myc antiserum, which also binds to PrA.
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 mt2Δ/mt2Δ 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 antiseraum (Fig. 1b). Therefore, neither of the higher-molecular-weight proteins recognized by the anti-ScHmt1 antiseraum is likely to be a type II PRMT. The putative enzyme or enzymes responsible for residual peaks in C. albicans hmt1Δ/hmt1Δ and mt2Δ/mt2Δ 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 might 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 steady-state localization and does not allow repression of Npl3-nuclear import in vivo.

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 DEHA0D50115g; 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 RGG-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 methylation affects the function of these proteins. In addition, the presence of residual methylarginine peaks in protein hydrolysates of hmt1Δ/hmt1Δ and mt2Δ/mt2Δ C. albicans suggests that future work may uncover another PRMT in this pathogenic fungus.

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ADDITION IN PROOF

The Npl3 protein as defined in assembly 20 of the C. albicans sequencing project (Fig. 4A) contains S8 more N-terminal amino acids than the assembly 19 Npl3 used in the experiments shown in Fig. 4B and C.

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