Pseudouridine synthase 7 impacts *Candida albicans* rRNA processing and morphological plasticity

Ethan S. Pickerill¹ | Rebecca P. Kurtz² | Aaron Tharp¹ | Paula Guerrero Sanz¹ | Munni Begum² | Douglas A. Bernstein¹

¹ Department of Biology, Ball State University, Muncie, IN 47306, USA
² Department of Mathematics, Ball State University, Muncie, IN 47306, USA

Correspondence
Douglas A. Bernstein, Department of Biology, Ball State University, Muncie, IN 47306. Email: dabernstein@bsu.edu

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Abstract

RNA can be modified in over 100 distinct ways, and these modifications are critical for function. Pseudouridine synthases catalyse pseudouridylation, one of the most prevalent RNA modifications. Pseudouridine synthase 7 modifies a variety of substrates in *Saccharomyces cerevisiae* including tRNA, rRNA, snRNA, and mRNA, but the substrates for other budding yeast Pus7 homologues are not known. We used CRISPR-mediated genome editing to disrupt *Candida albicans* PUS7 and find absence leads to defects in rRNA processing and a decrease in cell surface hydrophobicity. Furthermore, *C. albicans* Pus7 absence causes temperature sensitivity, defects in filamentation, altered sensitivity to antifungal drugs, and decreased virulence in a wax moth model. In addition, we find *C. albicans* Pus7 modifies tRNA residues, but does not modify a number of other *S. cerevisiae* Pus7 substrates. Our data suggests *C. albicans* Pus7 is important for fungal vigour and may play distinct biological roles than those ascribed to *S. cerevisiae* Pus7.

KEYWORDS

*C. albicans*, pseudouridine, pseudouridine synthase 7, pseudouridylation, Pus7, RNA modification, rRNA processing

1 | INTRODUCTION

RNA bases can be modified in over 100 distinct ways (Machnicka et al., 2013), and these modifications play critical roles in translation, transcription, and splicing (Hoernes & Erlacher, 2017; Roundtree, Evans, Pan, & He, 2017). Pseudouridine (Ψ) is one of the most prevalent RNA modifications and is found in all kingdoms of life (Ge & Yu, 2013). Pseudouridylation requires a pseudouridine synthase cleave the C–N glycosidic bond of a uridine in an RNA chain. The pseudouridine synthase then reattaches the uracil moiety at the C5 position, forming pseudouridine (Cerrudo, Ghiringhelli, & Gomez, 2014; Hammal & Ferre-D’Amare, 2006). The exposure of N1 following modification permits pseudouridine to participate in additional hydrogen bonds, which have been proposed to confer RNA structural stability (Ofengand, 2002). Pseudouridine is highly abundant in tRNA and the rRNA catalytic centre where it is proposed to play important roles in peptide bond formation (Bakin, Lane, & Ofengand, 1994). The amount of pseudouridylation varies among organisms. For instance, bacteria rRNA contain between five and 15 pseudouridines, whereas

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mammalian rRNA contains over 70 (Spenkuch, Motorin, & Helm, 2014). In addition, pseudouridine is found in small nuclear RNAs (snRNA), small nucleolar RNAs (snoRNA), and mRNA, (Carlile et al., 2014; Lovejoy, Riorand, & Brown, 2014; Schwartz et al., 2014; Yu, Ge, & Yu, 2011); however, the roles of pseudouridine in these RNA species are less clear.

Pseudouridylation is catalysed by pseudouridine synthases. The number of pseudouridine synthases varies by organism. Some bacteria encode a single pseudouridine synthase, whereas Saccharomyces cerevisiae encodes 10 pseudouridine synthases (Ofengand, 2002; Schwartz et al., 2014) that modify a variety of substrates. The mechanism by which pseudouridine synthases identify their targets varies. Cbf5, for instance, uses snoRNAs to recognize its targets (Lafontaine, Bousquet-Antonelli, Henry, Caizergues-Ferrer, & Tollervey, 1998; Watkins et al., 1998). All other S. cerevisiae pseudouridine synthases use a combination of RNA primary and secondary structure to identify their target residues. Some of these residues lie in consensus motifs, but the presence of a consensus motif does not ensure modification will occur (Schwartz et al., 2014). Some pseudouridine synthases target only a single residue or class of residues. For instance, pseudouridine synthase 5 (Pus5) only targets a single residue in the mitochondrial rRNA (Ansmant, Massenet, Grosjean, Motorin, & Branlant, 2000). S. cerevisiae pseudouridine synthase 7 (ScPus7) on the other hand modifies a wide variety of RNA substrates, including snRNA, tRNA, rRNA, and mRNA (Behm-Ansmant et al., 2003; Decatur & Schnare, 2008; Ma, Zhao, & Yu, 2003; Schwartz et al., 2014). Bacterial Pus7 homologues however modify only tRNAs (Kaya & Ofengand, 2003). Although PUS7 homologues are found in many fungal taxa, it is not known if such substrate promiscuity is conserved.

We characterized the lone Pus7 homologue encoded by the human fungal pathogen Candida albicans (CaPUS7). C. albicans is a member of the CTG clade of fungi that diverged from Saccharomyces roughly 170 million years ago (Massey et al., 2003). Little is known about the process of pseudouridylation in the CTG fungal clade, but genome-wide expression studies indicate CaPUS7 is induced during biofilm formation, a key fungal virulence factor (Murillo et al., 2005).

To examine the roles of CaPus7, we deleted CaPus7 from a clinical fungal isolate. Our data suggest CaPus7 is important for rRNA biogenesis and loss of CaPus7 results in changes to C. albicans virulence and fitness. Furthermore, we find CaPus7 and ScPus7 modify distinct substrates. Our data suggest although pseudouridine synthases are conserved between budding yeast taxa, their substrates and roles in vivo are not.

2 METHODS

2.1 Media

YPD: 1% yeast extract, 2% bacto-peptone, 2% dextrose, 0.15% L-tryptophan, 0.27-mM uridine. synthetic complete media (SC), yeast nitrogen broth-bovine serum albumin (YNB-BSA) (“Guide to yeast genetics and molecular biology,” 1991), and Spider media, (Liu, Kohler, & Fink, 1994).

2.2 CRISPR-Cas9-mediated C. albicans genome editing

pus7/pus7 C. albicans were generated using the method described in Vyas, Barrasa, and Fink (2015). Twenty-base pair guide sequences (Table S2) were cloned into vector pv1093 (Vyas et al., 2015), which encodes the Cas9 endonuclease as well as nourseothricin yeast and ampicillin bacterial drug resistance markers. One hundred-base pair repair templates (Table S2) introduced two consecutive stop codons and an EcoRI restriction site to PUS7 upon cleavage repair. Yeast cotransformed with guide and repair template were plated on YPD + Uri supplemented with 200 μg/ml of nourseothricin. Colonies were screened by PCR and EcoRI restriction digestion (Figure 1b). Strain names are listed in Table S3.

2.3 RNA sequencing and data analysis

RNA was extracted and purified using acid phenol. Five micrograms of total RNA was polyA-purified, cloned into libraries, and sequenced by Geneviz on a HighSeq 2500, run in high output mode, for single read of 50 bases. Reference genome SC5314 version A22-s07-m01-r20 was used for analysis. FASTA and gff files were separated by allele. The reference genome was indexed using Bowtie2, and reads were aligned to the indexed genome using Tophat (Trapnell et al., 2012). Alignments were organized into a matrix of read counts using the count function as part of the HTSeq package. Alignments that were ambiguous or not unique were not considered. Using the EdgeR package, genes were filtered from a matrix of counts on the basis of their counts per million reads (CPM). For each gene, a minimum of four libraries were required to have approximately 1.39 CPM to be included in the analysis. Libraries were normalized using EdgeR, and a negative binomial model was fitted for each gene to assess differences in expression level. Each condition had a corresponding coefficient in each model. A likelihood ratio test was used to assess if two given coefficients were significantly different. The cut-off to consider a gene for differential expression was a p value lower than .001 and an absolute log2FC of greater than 1.3.

2.4 Growth and filamentation

Assays were performed as previously described in (Evans, Smith, et al., 2018). Fourfold serial dilutions were plated using a pin replicator.

2.5 Galleria mellonella infection

Assays were performed as in (Evans, Smith, et al., 2018). Briefly, 235 mg ± 45 mg larvae were randomly assigned to receive 10-μl sterile PBS, wild type, or pus7/pus7 injections; 10⁷ cfu of wild type or pus7/pus7 log phase cultures grown at 37°C in YPD were washed.
twice and suspended in 1-ml sterile PBS. Larvae were incubated at 37°C, and survival was assessed daily by visual inspection. Experiments were discontinued after 7 days. Fifty larvae were subjected to each treatment, and data were assessed via Kaplan–Meier survival analysis and by the log rank test for multiple comparisons in R studio.

2.6 Drug resistance

Wild type or pus7/pus7 yeast were grown overnight in SC ("Guide to yeast genetics and molecular biology," 1991). One million colony-forming units of overnight cultures were suspended in 200-μl SC and plated on SC 2% agar. Fifteen microlitres of 1 mM fluconazole or 20 μl of 1 mM caspofungin was added to a sterile paper disc at the centre of each plate. Assays were repeated in triplicate, and disk image R was used to analyse the data as described in Gerstein, Rosenberg, Hecht, and Berman (2016).

2.7 Cell surface hydrophobicity

Cell surface hydrophobicity assays were performed as described previously (Hazen, Plotkin, & Klimas, 1986). Briefly, 10 wild type and pus7/pus7 YPD + Uri log phase cultures grown at 30°C or 37°C were washed and suspended in sterile PBS to yield 5 × 10⁶ cfu. OD600 values were recorded for each replicate. Three millilitres of each suspension was mixed with 1 ml xylene. Aqueous phase OD600 values were recorded for each replicate, whereas the aqueous phase of a 3:1 sterile PBS to xylene solution was used to blank the spectrophotometer. Percent change in OD600 value was calculated for each replicate, and data were assessed via boxplot in R studio. Percent change data were then analysed using analysis of variance Tukey's honestly significant difference test.

2.8 Polystyrene biofilm formation

Biofilm assays were performed as described previously with the following changes (Pierce et al., 2008). YPD + Uri log phase cultures were
diluted to $5 \times 10^6$ cfu per millilitre in RPMI with 5% serum, Spider, or YPD and added to a 96-well plate. Each treatment was repeated 16 times, and data were plotted in GraphPad Prism 7. Percent change data were then analysed using analysis of variance Tukey's honestly significant difference test.

2.9 | Northern blot

ITS2-specific probe was generated by PCR incorporating biotin-16-dUTP. ITS2 probe was purified using Zymo research gel purification kit. RNA isolation was performed as described above, and 5-μg RNA was loaded on a denaturing gel. Gel electrophoresis, transfer, and crosslinking were performed according to NorthernMax manual suggestions. Prehybridization, hybridization with ITS2 probe, washes, blocking, IRDye 800CW streptavidin incubation (1:10,000), and imaging were performed as described and recommended by the Odyssey Infrared Imaging System.

2.10 | Primer extension

RNA was purified as described above and N-cyclohexyl-N'-[2-morpholinoethyl]carbodiimide metho-p-toluenesulfonate (CMC) treatment was performed as described (Bakin & Ofengand, 1993) with the following modifications. One hundred millimolars of CMC was prepared in BEU buffer. Fifty micrograms of aliquots of RNA was mixed with 30 μl of 100-mM CMC in BEU or in 30 μl of BEU and incubated at 37°C for 20 min, followed by ethanol precipitation. RNA pellets were suspended in 40 μl of 50-mM Na2CO3 (pH 10.4) and incubated for 3 hr at 37°C. After incubation, RNA samples were ethanol precipitated and suspended in 10-μl H2O. Primer extensions were performed as described for avian myeloblastosis virus (AMV) reverse transcriptase (NEB M0277S) with the following modifications. One half microgram of CMC/Na2CO3-treated RNA, 0.5-mM dATP, 0.5-mM dCTP, 0.5-mM dGTP, 0.6-mM dTTP, 0.2-mM Biotin-16-dUTP, and 20-μM primer (Table S4) were combined in 10 μl, incubated at 65°C for 5 min, and cooled to 4°C. Two microlitres of AMV buffer, 2-μl AMV reverse transcriptase, and 6-μl H2O were added and incubated at 42°C for 1 hr and 80°C for 5 min. Unincorporated biotin-16-dUTPs were removed, and separation of extension products was performed on a 12% PAGE Urea gel as previously described (by National Diagnostics). Samples were run at 2 W for 20 min followed by 20 W for 2 hr. RNA was transferred to membrane using idEA Scientific GENIE Transfer Apparatus, and membrane was cross-linked at 120,000 μl. Blocking, IRDye 800CW streptavidin incubation (1:10,000), and imaging were performed as recommended by Odyssey Infrared Imaging System manual.

3 | RESULTS

To investigate the roles of CaPus7, C. albicans Cas9-mediated genome editing was used to introduce two stop codons at nucleotide 279 in C. albicans clinical isolate SC5314 (wild type); (Evans, Pickerrill, Vyas, & Bernstein, 2018; Vyas et al., 2015). Introduction of stop codons prematurely halts translation at amino acid 93 truncating CaPUS7 to eliminate the pseudouridine synthase domain (Figure 1a). PCR and restriction digestion (Figure 1b) confirmed five homozygous isolates (pus7/pus7).

RNA sequencing was performed to compare gene expression between wild type and pus7/pus7. A number of cell surface genes are down regulated in pus7/pus7 when compared with wild type (Tables 1 and S1). Gene Ontology analysis confirmed oligopeptide transporter genes are significantly down regulated in pus7/pus7 (Table 1, p value 4.79E-07). The gene that increases in abundance most in pus7/pus7 is KTI11, which has been proposed to play roles in rRNA wobble base modification (Huang, Johansson, & Bystrom, 2005). The transcripts exhibiting the next greatest increase in abundance in pus7/pus7 are Internal Transcribed Spacer 1 (ITS1) and Internal Transcribed Spacer 2 (ITS2). ITS1 and ITS2 must be cleaved from the 35S rRNA transcript before functional 5.8, 18S, and 25S RNA can form (Venema & Tollervey, 1999). Our data suggests rRNA processing intermediates are more abundant in pus7/pus7 and elimination of CaPus7 causes a defect in rRNA processing. The presence of increased unprocessed rRNA and rRNA processing intermediates in pus7/pus7, including 35S rRNA, was confirmed via northern blot probing for ITS2 (Figure 1c). Our results are consistent with previously described C. albicans rRNA processing intermediates (Pendrak & Roberts, 2011). Moreover, a number of cell surface protein transcripts were differentially regulated in pus7/pus7, and we tested if this led to a change in cell surface hydrophobicity and biofilm formation. Using a xylene hydrocarbon-partitioning assay, pus7/pus7 was found to be less hydrophobic than wild type (Figure 1d). In addition, pus7/pus7 less robustly formed biofilms compared to wild type (Figure 1e).

Next, reverse transcriptase primer extension was used to identify CaPus7 substrates (Figure 2a). In S. cerevisiae, Pus7 modifies a variety of RNA substrates, including snRNA, tRNA, rRNA, and mRNA (Behm-Ansment et al., 2003; Decatur & Schnare, 2008; Ma et al., 2003; Schwartz et al., 2014). We first tested if CaPus7 is required for modification of tRNA E (CUC), a modification that is conserved among fungi and metazoan (Behm-Ansment et al., 2003; Guzzi et al., 2018). CaPus7 is required for modification of residue U13 of tRNA E (CUC) as primer extension terminates at the expected 38 base product (Figure 2b). Additional Pus7-dependent primer extension termination occurred at extension products corresponding to residues U11 and U8. Next, we tested if CaPus7 is required for modification of U2 snRNA residues 36 or 57. These residues are analogous to sites modified by ScPus7 (Yu et al., 2011). We did not detect modification of either of these residues using primer extension (Figure S6). This suggests CaPus7 modifies some, but not all, of the same substrates as ScPus7.

### Table 1

| Transcripts with increased abundance in pus7/pus7 | IFO3, ITS1, ITS2, KTI11 |
| Transcripts with decreased abundance in pus7/pus7 | ALS2, CAN1, OPT2, OPT3, OPT4, OPT7, OPT9 |
To determine if loss of Pus7 leads to changes in phenotype, serial dilutions of pus7/pus7 and wild type were spotted on YPD or synthetic complete media. (Figures 3a and S1). Qualitative assessment of growth suggests pus7/pus7 grew more slowly than wild type cells at all temperatures and exhibited the most severe growth defect at 40°C. Many genes important for nitrogen import, including peptide transporters (Tables 1 and S1) were down regulated in pus7/pus7. We tested if pus7/pus7 cells were more susceptible to nitrogen limitation by growing them on synthetic complete media that contained BSA as a nitrogen source (YNB-BSA), but this did not exacerbate pus7/pus7 growth defects (Figure S2).

We next tested if the absence of Pus7 affects filamentation, a phenotype important for C. albicans virulence. Growth at 37°C on Spider agar robustly induced wild type filamentation but fails to induce pus7/pus7 filamentation after 8 days (Figure 3b). This filamentation defect was also observed at 25°C and 30°C (Figure S3). In liquid Spider media, we find pus7/pus7 filaments as robustly as wild type (Figure 3c). Because filamentation is critical for virulence, we tested if pus7/pus7 has reduced virulence in comparison with wild type in a Galleria mellonella infection model; 175 hours after injection, 75% of G. mellonella larvae treated with pus7/pus7 were still alive whereas only 20% of the worms treated with wild type were alive (p value <.001; Figure 4a). Nearly 100% of worms treated with PBS were alive after 175 hours. These data indicate that pus7/pus7 is significantly less virulent in the G. mellonella infection model.

Finally, we tested if the absence of Pus7 had an effect on antifungal drug resistance. A disk diffusion assay was performed with fluconazole, and the pus7/pus7 zone of inhibition was larger than wild type’s on day two of incubation at 37°C and 40°C (Figures 4b,c and S4). At 30°C, the zones of inhibition were very similar on day two (Figure 4d). This suggested that after plating, pus7/pus7 cells are initially more susceptible to fluconazole. However, after four days, pus7/pus7 exhibited pronounced trailing growth into the zone of inhibition, suggesting pus7/pus7 cells are better able to overcome the initial fluconazole susceptibility (Figures 4b and S4). Wild type did not display as robust trailing growth into the fluconazole zone of inhibition. Wild type and pus7/pus7 showed similar susceptibility to caspofungin, and neither strain exhibited trailing growth into the zone of inhibition (Figure S5). Pretreatment of wild type or pus7/pus7 with fluconazole led to lower virulence in wax moth larvae (Figure S7), and pus7/pus7 was not found to be more virulent than wild type after fluconazole pretreatment.
DISCUSSION

Absence of Pus7 is highly detrimental to *C. albicans* vigour, leading to slow growth, heat sensitivity, defects in filamentation, and decreased virulence. This is in contrast to *ScPUS7* knockout, which leads to moderate slow growth and heat sensitivity (Sinha et al., 2008). We find CaPus7 is essential for some tRNA pseudouridylation. tRNA pseudouridylation is important for translation (Klassen et al., 2016; Klassen & Schaffrath, 2017), and defects in these processes could be responsible for the observed phenotypes. tRNA E (CUC) Ψ13 is catalysed by CaPus7, and other Pus7-dependent stops at U11 and U8 were also observed. Pus7 may catalyse these additional sites of pseudouridylation, or U13 pseudouridylation may be required for U11 and U8 pseudouridylation via Pus7-independent mechanisms. ScPus7 often targets a consensus sequence, but this consensus site is not required. U8 lies within sequence RNUNΨAR, similar to the ScPus7 RSUNΨAR consensus sequence. Pus7-mediated modification sequence specificity has not been extensively studied outside of *S. cerevisiae*, and as such, requirements for site selection remain unclear in *C. albicans*.

A second molecular phenotype we observed was a disruption in rRNA processing. Multiple potential models explain how Pus7 absence could lead to defects in rRNA biogenesis. One potential model is direct Pus7-mediated modification of ITS1/ITS2 is important for rRNA processing and absence of such modification leads to increased ribosome precursors. The lone 8mer conserved between *C. albicans* ITS1 and ITS2 contains a ScPus7 consensus site (Schwartz et al., 2014). If Pus7 mediated-pseudouridylation at the consensus site is required for ITS1/ITS2 processing in *C. albicans*, pseudouridylated ITS1/ITS2 could be efficiently processed from the newly transcribed rRNA and degraded in wild type. In *S. cerevisiae*, ITS1 and ITS2 are processed and degraded cotranscriptionally or soon after transcription (Kos & Tollervey, 2010). This is consistent with our observation of fewer rRNA ITS2 containing precursors in wild type. However, in the absence of Pus7-dependent modification, ITS1 and ITS2 will not be modified. This will result in an increased abundance of rRNA precursors in *pus7/pus7* that contain nonpseudouridylated ITS2. We tested if CaPus7 modifies the ITS1 or ITS2 Pus7 consensus sites. No CMC-dependent transcription termination was observed in the ITS1 primer extensions (Figure S8). CMC dependent transcription termination does occur in ITS2, but this termination is nine or 10 bases upstream of the Pus7 consensus site and was not Pus7 dependent (Figure 2c). Termination upstream of the consensus site residue could mask CMC-dependent stoppage at the predicted Pus7 target, as reverse transcription would terminate before reaching the Pus7 consensus site. Our results are consistent with, but cannot confirm, this model.

Another potential model is Pus7 has an indirect effect on rRNA biogenesis. ScPus7 is promiscuous, and although we do not observe modification by CaPus7 at all analogous sites, it is possible that CaPus7 modifies a broad range of substrates at lower frequency or that modification of a small set of substrates has complex downstream
KTI11, an essential gene known to play roles in tRNA wobble base modification was the most up-regulated gene in \textit{pus7/pus7} (Segal et al., 2018). Significant increase in abundance of a key translational regulator such as Kti11 could have dramatic effects on proteins required for rRNA processing (Huang et al., 2005).

Pus7 homologues are found in a variety of fungi, but they have been only minimally characterized outside of \textit{S. cerevisiae}. Deletion of \textit{Schizosaccharomyces pombe} Pus7 (SpPus7) leads to a slight decrease in fermentative growth (Malecki & Bahler, 2016), but SpPus7 targets have not been investigated. Humans express two Pus7 homologues and tRNA-mediated modification by human Pus7 is thought to play important roles in stem cell differentiation (Guzzi et al., 2018). Furthermore, mutation of human \textit{PUS7} leads to developmental defects including short stature, microcephaly, and aggressive behaviour (de Brouwer et al., 2018). Our data indicate that the absence of \textit{C. albicans} Pus7 leads to severe defects in morphological plasticity and slow growth. In addition, our data suggest CaPus7 does not act on U2 snRNA, but does act on tRNA the primary target of mammalian and bacterial Pus7s (Guzzi et al., 2018; Kaya & Ofengand, 2003). Further biochemical characterization of Pus7 homologues is necessary to elucidate Pus7's contributions to health and disease.

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**ORCID**

Douglas A. Bernstein [https://orcid.org/0000-0001-8653-8562](https://orcid.org/0000-0001-8653-8562)

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SUPPORTING INFORMATION

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