Role of Ess1 in Growth, Morphogenetic Switching, and RNA Polymerase II Transcription in Candida albicans

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Abstract

Candida albicans is a fungal pathogen that causes potentially fatal infections among immune-compromised individuals. The emergence of drug resistant C. albicans strains makes it important to identify new antifungal drug targets. Among potential targets are enzymes known as peptidyl-prolyl cis/trans isomerases (PPIases) that catalyze isomerization of peptide bonds preceding proline. We are investigating a PPIase called Ess1, which is conserved in all major human pathogenic fungi. Previously, we reported that C. albicans Ess1 is essential for growth and morphogenetic switching. In the present study, we re-evaluated these findings using more rigorous genetic analyses, including the use of additional CaEss1 mutant alleles, distinct marker genes, and the engineering of suitably-matched isogenic control strains. The results confirm that CaEss1 is essential for growth in C. albicans, but show that reduction of CaEss1 gene dosage by half (Δ/+) does not interfere with morphogenetic switching. However, further reduction of CaEss1 levels using a conditional allele does reduce morphogenetic switching. We also examine the role of the linker α-helix that distinguishes C. albicans Ess1 from the human Pin1 enzyme, and present results of a genome-wide transcriptome analysis. The latter analysis indicates that CaEss1 has a conserved role in regulation of RNA polymerase II function, and is required for efficient termination of small nucleolar RNAs and repression of cryptic transcription in C. albicans.

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Introduction

Candida albicans causes life-threatening fungal infections in hospitalized patients [1–3]. C. albicans is a commensal organism found on the human mucosal surface and is generally harmless in healthy individuals [4,5]. However, C. albicans can cause systemic and sometimes fatal infections in immune-compromised individuals [6–8]. Life-saving therapies that require suppression of the immune system, e.g. organ transplantation and cancer chemotherapy, increase the risk for invasive candidiasis [9–12]. Premature infants, HIV-infected individuals, and individuals receiving prolonged intensive care treatment or antibiotic treatment are also vulnerable [13–16]. While potent antifungal drugs are available, the emergence of drug-resistant strains, especially against the widely used azole drugs is a growing problem [3,17–21].

One strategy to overcome drug resistance while producing synergistic effects is the use of combination therapies that target distinct intracellular pathways [22–27]. Toward this end, a number of different pathways are being investigated including those containing enzymes known as peptidyl prolyl cis-trans isomerases [28,29]. PPIases catalyze the isomerization of the peptide bond preceding prolines within protein substrates [30,31]. Three major families of PPIase have been described; cyclophilins, FK506-binding proteins (FKBPs) and parvulins (reviewed in [32]. All three families are conserved from yeast to humans. Inhibitors such as cyclosporin, which targets the cyclophilins, and FK506 or rapamycin, which target FKBPs, all show potent antifungal activity [33–36]. However, these drugs are also immunosuppressive via pathways that inhibit T-cell activation, an activity that could make individuals more vulnerable to fungal infections [37,38].

An alternative PPIase target might be Ess1, a founding member of the parvulin class of PPIase, which is structurally distinct from the cyclophilins and FKBPs [39,40] and whose human ortholog Pin1 is not known to be associated with the T-cell activation pathway. The first fungal Ess1 was discovered in Saccharomyces cerevisiae and shown to be essential [41]. Ess1 and Pin1 play critical roles in gene transcription by RNA polymerase II (pol II) [42–46]. Ess1 isomerizes peptide bonds within the carboxy-terminal domain (CTD) of Rpb1, the largest subunit of RNA pol II, and thereby controls binding and release of transcriptional co-factors [43,47–49]. The X-ray structures of the C. albicans Ess1 protein and its human ortholog, Pin1, have been solved [40,50,51], and while the enzymes show overall similarity, there are key differences including a large solvent-exposed alpha-helix within a structured
linker region that is present in the fungal enzyme but absent in the human enzyme. This helix has been hypothesized to play a role in fungal-specific functions, potentially by engaging in protein-protein interactions [50].

Homologs of Ess1 are found in all major pathogenic fungi that have been examined, including C. albicans [52], Candida glabrata (REFSEQ XP_445146), Cryptococcus neoformans [53], and Aspergillus nidulans (PinA) [54]. In C. albicans and A. nidulans Ess1 (PinA) is essential for growth [52,54]. In Cryptococcus neoformans, Ess1 is not essential for growth, but is required for expression of virulence factors melanin and urease and for virulence in a mouse model [53].

In previous work, we isolated the CaEss1 gene from C. albicans and showed it to be essential for growth in this organism using a novel temperature-sensitive mutant strategy [52]. Surprisingly, we found a heterozygous mutant strain (Caess1+/CaEss1) to be defective for filamentation in various inducing media leading us to conclude that CaESS1 gene dosage is important for morphogenetic switching [52]. However, several considerations led us to re-evaluate these findings. First, our CaEss1 dosage experiments used the URA4-blastor method [55], which has largely been superseded to avoid variations in virulence phenotypes due to expression of UR43 from ectopic loci [56,57]. Second, the importance of using reconstituted strains rather than parental strains as controls is now well-established [57,58]. Finally, sequencing of the C. albicans genome [59] revealed a gene, APE2, located very close to CaEss1, whose promoter might have been disrupted in our previous strain constructions.

We therefore re-examined our previous findings using newer information and more rigorous methods. The results showed that C. albicans Ess1 is essential for growth, but that in contrast to our previous report, CaEss1 heterozygous mutants did not show a defect in morphogenetic switching, and instead the defect was traced to a host cell mutation(s). Potential effects of UR43 marker gene placement were also ruled out. We also report results of a structure-function analysis of the CaEss1 linker α-helix, and describe a conditional-lethal readthrough allele of CaESS1 that will be useful for further studies. Finally, results of a transcriptome analysis using high-throughput RNA-sequencing suggests that C. albicans Ess1, like its counterparts in budding yeast and humans plays a role in regulating RNA polymerase II function.

Results and Discussion

Reduction of CaESS1 gene dosage does not affect filamentation phenotypes

In previous work, we constructed a C. albicans Caess1+/CaEss1 heterozygous mutant strain in the CAI4 (ura+/ura−) parent strain using the URA4-Blaster method [55] (Figure 1A). This strain, CaGD1, was defective for filamentation in Lee's, serum-containing and Spider media when compared to the “wild-type” control SC5314 [52]. The CAI4 (ura−) parent strain is a poorly-filamenting strain, presumably due to its uracil auxotrophy [60]. Instead, the parent strain of CAI4, clinical isolate SC5314, had been used as a control, because SC5314 is a uracil prototroph (ura+) and could therefore be compared to CaGD1 (ura−), even though SC5314 has two copies of UR43 and CaGD1 has only one copy.

Here, we generated a better control, isogenic to CAI4, but with one copy of the UR43 gene placed at the CaEss1 locus without disrupting it (Figure 1E). Surprisingly, this reconstituted control strain, R6 (ura−) (CaEss1/CaEss1), also did not filament on serum-containing medium or Spider medium even though both CaEss1 alleles were left intact (Figure 2E). Filamentation on Lee’s medium was also defective (data not shown). It is possible that the integrated UR43 construct in R6 somehow lowered CaESS1 expression levels in cis, but this was ruled out using quantitative reverse transcription real time PCR (qRT-PCR) and Western analysis, which shows the expected CaESS1 RNA and protein levels in the heterozygotes (CaGD1) and wild-type control (R6) strains (Figure 3). Thus, placement of the UR43 gene at the CaEss1 locus or some other defect, but not CaEss1 dosage, was likely responsible for the filamentation defect.

One possibility was that the UR43 gene placed at the CaEss1 locus interrupted the promoter region of the downstream gene, APE2. The APE2 gene encodes an aminopeptidase that is thought to be secreted through the cell wall [61]. The design of our UR43-based CaEss1 knockout and control constructs leaves only 15 bp of upstream sequence on one allele of APE2, leaving open the possibility that reduced expression of APE2 was responsible for the filamentation defect in both strains (Figure 1A, 1E). As described in a later section, neither UR43 nor APE2 expression was involved.

To independently confirm that CaEss1 gene dosage did not affect filamentation we took an approach that did not rely on UR43 markers or disrupt the APE2 promoter. We used a parent strain (SN87) in which HIS1 and LEU2 are used as selectable markers [62]. Extensive studies have shown that ectopic expression of these markers does not significantly affect the virulence functions [62]. Accordingly, we replaced one allele of CaEss1 (with HIS1) generating a heterozygous strain (the other allele was marked with LEU2) (Figure 1I). We also constructed a control strain in the same background, but leaving both CaEss1 alleles intact (Figure 1J). To avoid any expression defects in CaEss1 or the downstream APE2 gene, the constructs were designed to have a short direct-repeat sequence (163bp) flanking the markers such that the ES1 gene would have a total of 200 bp downstream of the coding sequence for transcription termination, and the APE2 gene would have 173bp of promoter sequence left intact (Figure 1I, J; see also Materials and Methods). As before, qRT-PCR and Western analysis shows the expected reduction by about half of the expression of CaESS1 mRNA and protein in heterozygous mutants (CaDS-B5.5) vs. the isogenic wild-type control (CaDS-B5) (Figure 3).

The Caess1/CaEss1 heterozygous mutant strain and its CaEss1/CaEss1 isogenic control showed no significant differences in filamentation phenotypes on inducing media (Figure 2I, J). In addition, no difference between these strains was observed in either germ-tube formation assays or in drug susceptibility growth assays using a number of commercially available antifungal drugs (data not shown). In summary, we find that the reduction of CaESS1 gene dosage by half does not affect major virulence-related phenotypes of C. albicans in-vitro. We conclude that one copy of CaEss1 is sufficient for growth and morphogenetic switching consistent with the finding that Ess1 in S. cerevisiae is present in excess under standard growth conditions [47].

Neither UR43 nor APE2 levels are responsible for the filamentation defect in our CAI4 isolate

If CaEss1 gene dosage was not responsible for the filamentation defects in our CAI4-derived strains, then what was responsible? To determine if ectopic UR43 at the ESS1 locus caused the filamentation defects in CaGD1 (Caess1+/CaEss1) and R6 (CaEss1/CaEss1) strains (Figure 2A,E), we created two additional sets of strains. In one set, the UR43 gene in both the CaGD1 and R6 strains were removed and replaced into the native UR43 locus (Figure 2B,C & F,G). In the other set, the UR43 gene was removed and placed at the RPS1 locus (Figure 2B,D & F,H). The RPS1 locus is commonly used to express UR43 and other genes to avoid positional effects [56,57,62]. We then used qRT-
Figure 1. Schematic representation of the strains used in this study. Strains A–H are made in the CAI4(*) background using URA3 as a selectable marker. Strains I–O are made in SN87 background with HIS1 and LEU2 selectable markers. The CaESS1 coding region and promoter region (pCaEss1) are shown in green; the APE2 gene is shown in purple. APE2 gene has two exons and the wavy line represents the intronic region. CaESS1 and APE2 are 210 bp apart in the genome (grey). The URA3 gene (blue) with the two flanking hisG direct repeats from S. typhimurium (yellow) is part of the genome.
PCR to compare the levels of expression of \textit{URA3} that resided at the \textit{CaESS1}, native (\textit{URA3} or \textit{RPS1}) loci. This was done for cells grown in selective (ura/deficient) media at 30°C and in 10% serum medium (10% FBS in YPD) at 37°C (Figure 4A,B). \textit{URA3} expression was essentially the same regardless of where the gene resided, thus ruling out position effects on \textit{URA3} expression as causing filamentation defects in our CAI4-derived strains. Moreover, placement of \textit{URA3} at different locations had no effect on expression of \textit{CaESS1} mRNA or protein (Figure 3).

Note, however, that all of the above strains carry one copy of the \textit{APE2} gene with a truncated or hisG-disrupted promoter (Figure 1A–F). To test whether reduced \textit{APE2} expression caused the filamentation defect, \textit{APE2} expression was measured in multiple strains (corresponding to Figure 1A–J) using qRT-PCR. Expression was measured in cells grown in selective media (30°C) and in serum-inducing medium (10% FBS in YPD, 37°C), and no significant differences in \textit{APE2} expression were detected (Figure 4C,D). These results suggest that the remaining copy of \textit{APE2} with an intact promoter (strains in Figure 1A–F) compensated for the loss of upstream sequences in the second copy, or that the transcription is regulated from an internal promoter. In experiments done in the SN87 background using a strain with both \textit{APE2} promoter regions truncated (\textit{5’ape2/5’ape2}) (Figure 1K), neither the expression of \textit{APE2} (data not shown) nor filamentation (Figure 2K) is affected. Results thus far indicate that \textit{APE2} expression is not correlated with the filamentation defect in CAI4-derived strains.

Finally, to characterize our parental CAI4 isolate, which we now concluded was suspect, we placed \textit{URA3} at the native locus or at the \textit{RPS1} locus and tested its ability to filament. Analogous CAI4-derived strains (e.g. CAI12, Figure 2R) made in other laboratories using similar approaches [56,63,64] were able to filament, but the strains derived from our CAI4 isolate were not (Figure 2P,Q). Thus, not all CAI4 isolates behave similarly under inducing conditions, and we suspect that the isolate we used previously had acquired a background mutation(s) that resulted in a loss in the ability to undergo morphogenetic switching. These results explain the difference between our current results and those reported in Devasahayam et al., [52] with respect to \textit{CaESS1} gene dosage and filamentation phenotypes. They may also explain the reduction in organ load in mice injected with SC5314 vs. CAI4-derivatives with mutations in \textit{CaESS1} [50].
CaESS1 is essential for growth in C. albicans

Previously, a novel temperature-sensitive (ts) approach was used to demonstrate that CaESS1 is essential for C. albicans viability [52]. In this approach one allele of CaESS1 was deleted and the second allele was replaced with a form of CaESS1 engineered to be conditional. Specifically, a substitution in a critical histidine residue in the C. albicans Ess1 active site (H171R) was generated based on a ts-mutation (ess1H164R) well characterized in S. cerevisiae [42]. Prior to its use in C. albicans, the CaESS1H171R allele was tested in complementation assays in S. cerevisiae and confirmed to be conditional; cells grew at 25°C and 30°C, but not at 37°C. When this allele was integrated into C. albicans CAI4, the resulting strain (Caess1D/Caess1H171R) strain was ts-lethal: it grew at 30°C but not at 40°C, demonstrating that Ess1 is essential in C. albicans.

Here we sought to confirm, using this ts-strategy, that Ess1 is essential in another strain background, using a marker system other than URA3. Despite repeated attempts in the SN87 background, we were unable to generate a Caess1D/Caess1H171R strain, consistent with CaEss1 being essential and suggesting that in this strain background the Caess1H171R allele cannot support growth. However, this is a negative result, so we took another approach.

We noted that the analogous S. cerevisiae ess1H164R protein has <0.01% the catalytic activity of wild-type protein [47] and decided to generate an additional allele, again based on prior work in S. cerevisiae [42]. The allele we generated alters the termination codon (TAA to TGC), resulting in translational readthrough. In S. cerevisiae, analogous mutations resulted in longer, fusion proteins that rendered the corresponding strains ts (X. Wu and S. Hanes, unpublished). The C. albicans strain we generated here, CaDS-C (ess1D/Caess1TGC; Figure 1L), which contains mutations in the stop codon (TAA to TGC) in CaEss1, encodes a protein that is larger than normal and is thermolabile at 42°C (Figure 5A). The level of mutant protein is only slightly reduced after two hours at 37°C (data not shown).

Figure 3. CaESS1 is expressed at reduced levels in heterozygous mutants as expected. (A) Quantitative reverse transcription PCR (qRT-PCR) shows CaESS1 mRNA expression levels in the indicated strains. (B) Western blot analysis showing the expression of CaEss1 protein in the indicated strains. A total of 3 μg of protein was used per lane. The blot was probed using anti-CaEss1 polyclonal antibody at a 1:500 dilution. CaEss1 is ~19 kD. Strains correspond to those shown in Figures 1 and 2: (ESS1/ESS1) URA3 at CaESS1 is R6; (ess1D/ESS1) URA3 at CaESS1 is CaGD1; (ESS1/ESS1) HIS1/LEU2 is CaDS-B5; (ess1D/ESS1) HIS1/LEU2 is CaDS-B5.5. doi:10.1371/journal.pone.0059094.g003
We compared the growth at different temperatures of the termination-mutant strain (CaDS-C) to an isogenic, reconstituted control strain in which the TGC readthrough codon was changed back to a TAA termination codon (CaDS-FC; Caess1<sup>d</sup>/CaES-S<sup>TAA</sup>). Both strains grew at lower temperatures (30°C and 37°C), but only the wild type (reconstituted) strain grew at 42°C (Figure 5B). The results show that the Caess1<sup>ts(TGC)</sup> allele is unable to support sustained growth at the restrictive temperature demonstrating that CaEss1 is essential for growth in the SN87 strain background. Thus, using a distinct conditional allele (Caess1<sup>TGC</sup>) in a different strain (SN87) with different markers (HIS1, LEU2), we confirm that CaEss1 is essential in <i>C. albicans</i>.

A large reduction in Ess1 levels reduces filamentation

In the course of characterizing the ts-lethal growth defect of the readthrough mutant strain (CaDS-C, Caess1<sup>d</sup>/CaEss1<sup>TGC</sup>), we noticed that at 37°C there is a reduction in filamentation relative to the control strain (CaDS-FC; Caess1<sup>d</sup>/CaEss1<sup>TAA</sup>) under inducing conditions. This reduction is readily apparent for cells grown for 4 days on solid Spider medium (Figure 5C, upper panels). Even after 7 days, colonies were unwrinkled compared to controls (data not shown). To confirm this result, we examined cells grown in liquid Spider medium for 2 hrs. Again, we find that the readthrough mutant (Caess1<sup>d</sup>/CaEss1<sup>TGC</sup>) is reduced in germ-tube formation relative to reconstituted control strain (Caess1<sup>v</sup>/CaESS1<sup>TAA</sup>) (Figure 5 C. ). This is an intriguing result because we do not observe a growth-rate defect in the readthrough mutant when grown in standard (non-inducing) medium, even at 37°C. It seems likely therefore that the levels of Ess1 protein in this mutant strain are sufficient for standard growth but not for specialized functions such as the induction of filamentation pathways. This is consistent with findings in <i>S. cerevisiae</i> showing that Ess1 is present in vast excess in cells growing in standard media (YPD, CSM), but that under various stress conditions, a significant reduction in Ess1 levels rendered cells unable to grow [47].

**Figure 4.** <i>URA3</i> and <i>APE2</i> mRNA expression levels are not significantly altered in heterozygous <i>ESS1</i> mutants and controls. qRT-PCR showing a quantitative measurement of <i>URA3</i> and <i>APE2</i> mRNA expression in the indicated strains. (A, C) Cells were grown at 30°C in complete synthetic medium (CSM), CSM minus uracil, or CSM minus histidine and leucine as appropriate for each strain. (B, D) Cells were grown at 37°C in serum-containing medium (10% FBS in YPD). Strains are as described in legend to Figure 3.
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help understand these key structural differences, we generated two mutations in the CaEss1 protein. First, we replaced residues 37–67 in C. albicans Ess1 protein with the flexible linker found in human Pin1 (residues 40–54), effectively generating a linker-swapped mutant. Second, we substituted three residues along the surface of the α-helix that would drastically alter the charge pattern it displays (E44K, A51D, and K54E; Figure 6A). As an initial test of protein function we tested their ability to complement a S. cerevisiae ess1Δ mutant (ess1ΔH164R) at non-permissive temperature (Figure 6B) and to complement an ess1Δ mutant using a plasmid-shuffle strategy (Table 1). The C. albicans linker-swapped mutant was unable to complement in either assay, whereas the helix mutant fully complemented in both assays.

To confirm that the mutant C. albicans Ess1 proteins were expressed and stable in S. cerevisiae, we performed Western blot analysis (using antibodies to C. albicans Ess1). The linker-swapped mutant was present at very low levels (Figure 6C), likely explaining its failure to complement in the functional assays. Due to its presumed instability, the linker-swapped mutant was not used for further studies. In contrast, the helix mutant was expressed at much higher levels, indicating the protein is stable consistent with its functionality (Figure 6C). That the helix mutant complements S. cerevisiae ess1Δ mutants suggests it performs the essential catalytic function required for growth in this organism. This result is not entirely surprising given that the human and other vertebrate orthologs lacking this α-helix also rescue growth in S. cerevisiae [66]; Wilcox & Hanes, unpublished]. We interpret this result to mean either the α-helix is required solely for specialized functions, e.g. specific protein-protein interactions in C. albicans that might be lacking (or not revealed) in S. cerevisiae, or that it is not important for growth under standard conditions.

To further test the importance of this α-helix, we introduced the helix mutant (hm) alleles into C. albicans cells using the SN87 parent strain. Two strains CaDSE-5 (hm/hm) and CaDSE-5.10 (Caess1Δ/hm) were constructed along with their respective isogenic controls. Both strains, CaDSE-5 and CaDSE-5.10 (Figure 1N, O) were tested for filamentation, germ tube formation and anti-fungal drug susceptibility profiles. No significant differences were seen in any of these assays (Figure 2N, O, and data not shown), suggesting the linker helix of CaEss1 is not important for morphogenetic switching or drug sensitivity in cultured cell assays. Thus, the prediction that the linker helix is important for fungal-specific functions has not yet been demonstrated. It is also possible that the triple mutation is not severe enough to inactivate its putative function(s), or that studies in animals models are needed to detect functional consequences of these mutations.

A conserved role for CaEss1 in RNA polymerase II transcription

Orthologs of CaEss1 in budding yeast (Ess1) and humans (Pin1) regulate RNA pol II transcription [42,45,46]. In yeast, Ess1 coordinates the recruitment of co-factors required for proper RNA synthesis and processing [43,49]. In this role, ScEss1 is required for efficient termination of at least two classes of RNA pol II products, small non-coding RNAs, e.g. small nucleolar RNAs (snoRNAs), and protein-coding mRNAs. ScEss1 is also important for repressing the transcription of cryptic unstable transcripts (CUTs) [49]. In S. cerevisiae ess1Δ mutants, CUTs are detected throughout the genome, many of which are the same as those detected in RNA-decay mutants [43]. To determine whether CaEss1 plays an analogous role in C. albicans, we carried out a whole-genome transcript analysis in wild-type and CaEss1 mutant strains using high-throughput RNA sequencing (RNA-seq). The strains and conditions used are summarized in Table 2. A large
number of mapped sequence reads were obtained (Table 2), indicating that our RNA libraries were of high quality.

For each mutant and control pair analyzed, there was a significant change in the level of expression for between 2–4% of other genes in C. albicans (non-coding transcript (CUT) (e.g. SNR32a, SNR43a, SNR43a, and SNR189) [67]. Primary transcripts of the other major class of snoRNAs (box C/D) are often encoded within other genes in C. albicans and were not examined here [68–70].

To determine whether CaEss1 represses cryptic transcription we used IGV to compare the transcription profiles of the ts-mutant and its isogenic control (ess1Δ/ESS1TAA) (data not shown) to find that about one in five show evidence of readthrough as revealed by RNA-seq data. These genes included SNR32a, SNR8a, SNR32a, SV40a, and SNR189 [67]. Primary transcripts of the other major class of snoRNAs (box C/D) are often encoded within other genes in C. albicans and were not examined here [68–70].

Each of the five H/ACA-class genes listed above contained an abundance of sequence reads (>30, but more often in the hundreds) immediately downstream of the putative termination site in the mutant background, suggesting possible transcription readthrough. The IGV profiles for three of these genes are shown (Figure 7, A–C). RNA sequence reads in the forward direction (red) and reverse direction (blue) are indicated as well as the overall number of reads (grey). For the control (ess1Δ/ESS1TAA) (Figure 7A–C) and wild-type (ESS1/ESS1) (data not shown) strains, the large number of reads is likely to reveal the actual position of the snoRNA transcription units (dotted grey bars) more accurately than sequence annotations (solid bars) [68–70]. The IGV also reveals examples of increased transcript abundance in the mutant cells for some snoRNAs, e.g. SNR32a, SNR43a, and SNR189 (Figure 7B,C), and a protein-coding gene (ORF19.1968) and a non-coding transcript (CUT) (Figure 7B).

The number of snoRNA readthrough transcripts, we carried out qRT-PCR (Figure 7D). cDNA synthesis was done using strand-specific primers, so only transcripts in the forward (‘sense’) direction relative to the snoRNA transcription units would serve as templates. Primer sets for amplification consisted of one primer within the snoRNA gene, and the other downstream of the putative termination site (Figure 7A–C), Therefore, only extended (readthrough) transcripts would be detected. The results show a 2–15 fold increase in the amount of readthrough transcription in the CaEss1 ts-mutant cells relative to isogenic control cells (Figure 7D). In summary, both the RNA-seq and qRT-PCR data indicate that CaEss1 is important for efficient termination of at least some snoRNA genes in C. albicans.

To determine if CaEss1 represses cryptic transcription we used IGV to compare the transcription profiles of the ts-mutant and its isogenic control, focusing on the intergenic regions along all of the chromosomes. In the mutant cells, intergenic transcription was rampant, with large amounts detected on all eight chromosomes. Examples are shown from chromosomes 2, 4 and 8 (Figure 8A–C). Intergenic transcripts that lie between divergent genes are probably CUTs, since they cannot be due to readthrough transcription from neighboring genes (e.g. Figure 8A). Using both short and long qRT-PCR to characterize other potential CUTs revealed that only the short products (marked with an asterisk in Figure 8B,C) were amplified (Figure 8D, and data not shown), indicating the RNA-seq reads are not due to readthrough transcription. These data suggest that in CaEss1 mutants, cryptic promoters are activated and/or cryptic RNAs are stabilized. CUTs from each of the eight chromosomes were easily detected by
work caused the defect in filamentation. Thus, like other studies a background mutation(s) in the isolate of CAI4 used in the prior confirmed in the present study. The most likely explanation is that genetic switching, which is required for virulence in vivo.

C. albicans growth in liquid CSM minus his. After 3 days, individual colonies were patched onto CSM minus his trp plates, and replica-plated to CSM minus ura (for Ura+), 1mg/ml 5-FOA (for Ura−), and CSM minus his trp (for total patch number) and scored for uracil prototrophy after 1 day. For the helix substitution mutants, three independent clone isolates of helix substitution plasmid, pDS413(sw) were tested. For the linker-swapped mutants, two independent clone isolates of plasmid, pDS413(pm) were tested.

Conclusions
In this study, we showed that the ESS1 gene is essential for growth in C. albicans. This result independently confirms earlier findings [52], but used more rigorous methods that included better controls and considered genomic sequence information that was not previously available. In contrast, the prior finding that reducing gene dosage by half (ess1Δ/ESS1) prevents morphogenetic switching, which is required for virulence in vivo [60], was not confirmed in the present study. The most likely explanation is that a background mutation(s) in the isolate of CAI4 used in the prior work caused the defect in filamentation. Thus, like other studies [72,73], our results make clear the importance of reconstituted isogenic controls, appropriate marker gene systems, and the dangers of isolate-to-isolate variability between supposedly identical strains. The results of transcriptome analysis of CaEss1 mutants support the idea that regulation of RNA polymerase II by Ess1 is conserved between the evolutionarily distant species S. cerevisiae and C. albicans. Further studies will be needed to determine whether transcriptional defects are responsible for growth and morphogenetic switching phenotypes in CaEss1 mutants, and whether the mechanism by which Ess1 functions in transcription in C. albicans is similar to that in S. cerevisiae [43,49]. With respect to C. albicans Ess1 as an antifungal drug target, data from this study suggest that elimination or strong inhibition of Ess1 enzyme activity will prevent growth. Given that Ess1 is essential not only in C. albicans, but also in A. nidulans [54], and is required for virulence in C. neoformans [53], it is possible that inhibitors of Ess1 could potentially be developed into broad-spectrum antifungal agents.

Materials and Methods
C. albicans strains, media, and transformation
All parent strains were grown in YPD media while the engineered derivative strains were grown in their respective selective media [74], unless otherwise specified. To select for URA3 loop-out events, cells were plated on YPD supplemented with 1mg/ml 5-FOA [75]. In addition to the descriptions below, please refer to the tables for details on primers (Table 4), plasmids (Table 5) and strains (Table 6) used in this study.

| Strain    | Harvest Conditions | # of Mapped Reads |
|-----------|--------------------|-------------------|
| At Weill Cornell Medical College, NY | 37°C serum induction (2 hr) | 22,000,000 |
| CaDS-B5 (ESS1/ESS1) | 37°C serum induction (2 hr) | 22,000,000 |
| CaDS-C (ess1Δ/ess1)^TS^- mutant | 30°C->42°C temp.shift (1 hr) then serum induction (2 hr) | 28,000,000 |
| CaDS-FC (ess1Δ/ESS1)^TS^- control | 30°C->42°C temp.shift (1 hr) then serum induction (2 hr) | 21,000,000 |
| At Centennial Biosciences, CA | 37°C serum induction (2 hr) | 22,000,000 |
| CaDS-B5 (ESS1/ESS1) | 37°C serum induction (2 hr) | 22,000,000 |
| CaDS-B5.5 (ess1Δ/ESS1) | 37°C serum induction (2 hr) | 21,000,000 |

Table 1. Function of C. albicans Ess1 linker mutants in S. cerevisiae.

| Strain                              | # Patches | Ura(+) | Ura(−) | % Plasmid Loss | Interpretation          |
|-------------------------------------|-----------|--------|--------|----------------|-------------------------|
| pRS413 (neg) control                | 400       | 400    | 0      | 0              | No complementation      |
| pCaESS1 (WT) (pos) control          | 216       | 144    | 72     | 33             | Control level of complementation |
| pCaESS1 linker-swapped mutant       | 324       | 208    | 116    | 36             | Full complementation    |
| pCaESS1 helix substitution mutant   | 400       | 400    | 0      | 0              | No complementation      |

Host strain (MATa ura3-1 leu2-3, 112 trp1-1 can1-100 ade2-1 his3-11, 15 [pHi+] ess1ATR1+YEpESS1) is an ess1ATR1 mutant of S. cerevisiae (Wu and Hanes, 2000, unpublished) covered by a 2μM, URA3 plasmid expressing ESS1 (YEpEss1). Cells were transformed with the indicated plasmids (all 2μM, HIS3) and plated on complete synthetic media (CSM) minus uracil (ura), histidine (his) and tryptophan (trp). Colonies were picked and passaged (20 ul into 3ml) for three successive overnights in liquid CSM minus his. After 3 days, individual colonies were patched onto CSM minus his trp plates, and replica-plated to CSM minus ura (for Ura+), 1mg/ml 5-FOA (for Ura−), and CSM minus his trp (for total patch number) and scored for uracil prototrophy after 1 day. For the helix substitution mutants, three independent clone isolates of helix substitution plasmid, pDS413(pm) were tested. For the linker-swapped mutants, two independent clone isolates of plasmid, pDS413(sw) were tested.

Table 2. Summary of RNA sequencing experiment.

qRT-PCR (e.g. Figure 8D), indicating that CUT expression is widespread in CaEss1 mutant cells.

Many putative CUTs were also found within open reading frames, often in the antisense direction. One example is a potential antisense CUT within the open reading frame of FGR46 (Figure 8E), a gene implicated in filamentation by a transposon insertion screen[71]. The reverse orientation of this CUT relative to the FGR46 ORF was confirmed by strand-specific qRT-PCR (Figure 8F). Thus, it is possible that under conditions where Ess1 levels are strongly reduced, CUT induction might enhance or suppress expression of genes important for virulence. Additional analyses will be needed to fully explore the information in the RNA-sequencing datasets and determine the effect(s) of CaEss1 mutation on expression of virulence-related genes. Thus far, our findings are consistent with a conserved role for CaEss1 in transcription by RNA polymerase II.
tions were performed according to the lithium acetate protocol [76].

*C. albicans* strain construction

**Insertion of the *URA3* gene at different genomic positions.** For re-evaluating the *C. albicans* heterozygous *Caess1* mutant (*CaGD1*)[52] along with its control strain, R6 (*CaESS1/CaESS1*), the *URA3* gene was removed from the *CaESS1* locus using 5-FOA mediated loop-out events to create uracil auxotrophs *CaGD2* (*Caess1/ Caess1*) and R6* (*CaESs1/CaESs1*) respectively. One copy of *URA3* was integrated at the native *URA3* locus by digesting pLUBP plasmid [77] with BglII and PstI restriction enzymes and transforming into the above strains (*CaGD2* and R6*). Similarly, one copy of *URA3* was integrated into the *RPS1* locus in these strains by digesting Cip10 plasmid [78] with StuI restriction enzyme and transforming. *URA3* was also integrated into the parent strain *CaAT* using the same pLUBP and Cip10 plasmids. (*) indicates a strain that may have acquired one or more mutations affecting filamentation as per this study.

**Isogenic wild-type control strain, *CaDS-B5 (ESS1/ESS1)*.** Plasmid pDS426(pm) is a derivative of pRS426 that carries the *CaESs1* promoter region (−255 to 0), *CaESs1* ORF (with helix point mutations) and *CaESs1* termination region (+535 to +755) between EcoR1 and BamH1 sites. The *LEU2* marker (from plasmid pSN40) and the *HIS1* marker (from plasmid pSN52) [62] was cloned into plasmid pDS426(pm) between the BamH1 and NotI sites. These plasmids were named pDS(b)Leu and pDS(b)His, respectively. Because in the *C. albicans* genome, *APE2* is just 210 bp downstream of *ESS1*, a 163 bp sequence (+572 to +735) was repeated on either side of the selectable markers to allow enough sequence for *CaESs1* termination and for the 5’ *APE2* promoter region. This was done by amplifying the downstream sequence of *CaESs1* (+572 to +1265) with NotI ends (primers OW1075 and OW1081), digesting and inserting into pDS(b)Leu and pDS(b)His, thus positioning the NotI-NotI downstream sequence after the markers. The resulting plasmids are pDS(c)Leu and pDS(c)His.

The pDS(c)Leu and pDS(c)His plasmids were digested with SacI and serially transformed into SN87 [62]. In the first step, one *CaESs1* allele was marked with the *LEU2* gene to generate strain *CaDS-B0.5* (**ESS1: LEU2/ESS1**). In the next step, the second allele of *CaESs1* was marked with *HIS1* gene to generate a *Leu*-*His* control strain *CaDS-B5* (**ESS1: LEU2/ESS1: HIS1**). Allele-specific PCR (primer OW221 with OW769 or OW770) and DNA sequencing was used to identify clones in which the integrating DNA recombined at positions that generated the wild-type *CaESs1* ORF, *i.e.* that excluded the helix mutations. The gene modifications were verified using junction-PCR and Southern blot hybridization (data not shown).

**Heterozygous mutant strain, *CaDS-B5.5 (ess1o/ESS1)*.** The NdeI site in the backbone of the plasmid, pDS(c)His was removed by digestion with NdeI, Klengow treatment and religation, to form pDS(d)His. The start codon (ATG) of *CaESs1* ORF in pDS(d)His was then converted to an NdeI site with the Change-IT Multiple Mutation Site Directed Mutagenesis Kit (USB) using the mutagenesis primer OW1099. The resulting pDS(c)His plasmid was digested with NdeI and SpeI (site 591 bp upstream of the *HIS1* marker), Klengow treated and religated to generate a plasmid that lacked the *ESS1* ORF sequence. The resulting plasmid, pDS(x)His was digested with SacI and transformed into strain *CaDS-B0.5* (**ESS1: LEU2/ESS1**) to generate a heterozygous mutant, *CaDS-B5.5 (ESS1: LEU2:ess1o/ HISS1)*. The gene modifications were verified using junction-PCR and Southern blot hybridization (data not shown). In plasmid, pDS(x)His the *HIS1* marker (from the pSN52 plasmid) was left with a 591 bp promoter region. An additional 393 bp sequence that was upstream of the promoter region of *HIS1* is removed in this construct. This had no affect on *HIS1* expression by qRT-PCR (primers OW1415 and D25) in comparison to strain *CaDS-B5 (ESS1/ESS1)* (data not shown).

**Temperature-sensitive strain, *CaDS-C (ess1o/ess1TGC)* and isogenic control.** The plasmid pDS(x)His was digested with SacI and transformed into SN87 to generate *CaDS-B5.5 (ess1o/ESS1)*, which is auxotrophic for *LEU2*. The NdeI site of the vector portion in plasmid pDS(c)Leu was removed (as done for pDS(c)His), to form pDS(d)Leu. Next, the start codon (ATG) and the stop codon (TAA) of *CaESs1* in plasmid pDS(d)Leu were changed to an NdeI site and a SphI site (primers OW1099 and OW1100, respectively) in two steps, to first form plasmid pDS(c)Leu and then plasmid pDS(f)Leu, respectively. The wild-type *CaESs1* sequence was amplified (primers OW1354 and OW1355) with flanking NdeI and SphI sites, digested and inserted into the same sites of pDS(c)Leu to give pDS(g)Leu. The SphI site changes the stop codon from TAA to TGC. The pDS(g)Leu plasmid was digested with SacI and transformed into *CaDS-B5.5 (ess1o/ESS1)* to make a prototrophic strain, *CaDS-C (ess1o/ess1TGC)*. This strain was verified using junction-PCR and Southern blot hybridization (data not shown). The control strain was generated by a novel strategy. About a 900 bp PCR fragment amplified (using primers OW216 and OW1251) from a wild-type background containing the normal termination codon (TAA) was introduced into CaDS-C (*ess1o/ess1TGC*). Transformants capable of growth at 42°C were selected, analyzed by PCR amplification and DNA sequencing of the *CaESs1* alleles and

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**Table 3. Changes in gene expression based on RNA-sequencing results.**

| Test Strain (& growth conditions) | Relative to | Total affected genes (%) | Log₂ fold-change | P value |
|----------------------------------|-------------|--------------------------|------------------|---------|
| **CadS-C (ess1+/ess1TGC)** 30°C→42°C temp.shift (1 hr), serum induction (2 hr) | CadS-FC (ess1+/ESS1TGC) 30°C→42°C temp.shift (1 hr), serum induction (2 hr) | 2.24 | 6.4–0.99 | 0–0.002 |
| **CadS-C (ess1+/ess1TGC)** 30°C→42°C temp.shift (1 hr), serum induction (2 hr) | CadS-B5 (ESS1/ESS1) 37°C serum induction (2 hr) | 3.43 | 7.5–1.3 | 0–0.003 |
| **CadS-B5.5 (ess1+/ESS1TGC)** 37°C serum induction (2 hr) | CadS-B5 (ESS1/ESS1) 37°C serum induction (2 hr) | 3.3 | 3.4–0.41 | 0–0.0017 |

The log₂ fold changes are based on results from the Cuffdiff program in the Galaxy server. Cuffdiff was used to select significant gene expression changes depending on whether the p value was greater than the allowed false discovery rate after Benjamini-Hochberg correction for multiple-testing. The highest and lowest log₂ fold changes of the genes considered to be significantly differentially expressed in each group are documented in the table.

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the mutation of TGC to TAA was confirmed. The resulting prototrophic strain CaDS-FC (ess1d/ESS1TAA) produced Ess1 protein of the normal size (19 kDa) and was non-ts.

**Linker-helix mutations in CaEss1.** PCR overlap extension (with primer sets OW748-OW1224, OW749-OW1233, and OW1224-OW1233) was used to generate a fragment that encodes CaEss1 protein bearing the linker region of human Pin1 (codon optimized for *C. albicans* [79]. This linker-swap fragment was digested with EcoRI and BamHI sites and cloned into the same sites of pRS426 vector to form pDS426(sw). A similar strategy was used to generate a CaEss1 mutant bearing three amino acid substitutions (E44K, A51D, and K54E) in the linker helix (using primers OW750 and OW751). The helix mutant fragment was cloned into pRS426 vector to form pDS426(pm).

**Helix mutants, CaDS-E (ESS1/hm), CaDS-E5 (hm/hm), and CaDS-E5.10 (ess1d/hm).** To construct CaDS-E (ESS1/hm) strains, the plasmid, pDS(c)Leu was digested with SacI and transformed into SN87. Allele-specific PCR and DNA sequencing was used to verify the clones that recombined to include the helix mutations (Caess1hm). This strain is auxotrophic for HIS1. To construct prototrophic CaDS-E5 (hm/hm) strains, pDS(c)His was digested with SacI and transformed into CaDS-E (ESS1/hm). To construct CaDS-E5.10 (ess1d/hm) strains, pDS(x)His was digested with SacI and transformed into CaDS-E(ESS1/hm). Mutants were verified using junction-PCR and Southern blot hybridization (data not shown).

**APE2 promoter mutant, CaDS-51B-2 (5’ape2/5’ape2).** A Not1-Not1 PCR fragment (primers D31 and OW1081) was cloned into plasmids pDS(b)Leu and pDS(b)His, to form plasmids...
Figure 8. RNA-sequencing reveals widespread cryptic transcription in CaEss1 ts-mutant cells. Results are shown using Integrated Genome Viewer as described in the legend to Figure 7. The positions of cryptic unstable transcripts (CUTs) are identified on representative chromosomes (A–C). (D) Results of qRT-PCR to detect CUTs (examples for each chromosome) expressed as a fold-change over wild-type (CaDS-B5) (ESS1/ESS1), normalized to ACT1. The approximate positions of the qRT-PCR products in chromosome 2, 4 and 6 samples in (D) are indicated by the short green arrows marked by an asterisk (*). The longer products indicated by long green arrows (B, C) did not amplify, indicating that the CUT signal is not likely due to a readthrough product from nearby open reading frames (ORF). cDNA synthesis was primed using a mixture of random hexamers and oligo(dT). Cell growth conditions were as listed in Table 2 (shift to 42°C, then serum induction at 42°C). (E) Prominent CUT identified within open reading frame of FGR46, which has been implicated in filamentation. (F) CUT is in reverse orientation relative to the FGR46 ORF as shown by strand-specific cDNA synthesis followed by qRT-PCR. Results are expressed as fold-change of ts-mutant (CaDS-C) over isogenic control (CaDS-FC), normalized to ACT1. Cells were serum-induced at 37°C. cDNA synthesis was primed using strand-specific primers that would reverse transcribe forward (UP044) or reverse (UP043) transcripts. For details, see Materials and Methods.

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| Primers | Description |
|---------|-------------|
| OW1075  | Forward primer to amplify sequence downstream of CaESS1 (+572 to +1265) with NotI ends |
| OW1081  | Reverse primer to amplify sequence downstream of CaESS1 (+572 to +1265) with NotI ends |
| OW221   | Reverse primer for allele-specific PCR |
| OW769   | Forward primer to detect helix mutations |
| OW770   | Forward primer to detect wild-type CaEss1 |
| OW1099  | Mutagenesis primer to introduce NdeI site at ATG (CaESS1 start codon) |
| OW1415  | Forward primer to test expression of HIS7 |
| D25     | Reverse primer to test expression of HIS7 |
| OW1100  | Mutagenesis primer to introduce Sphi site at TAA (CaESS1 stop codon) |
| OW1354  | Forward primer to amplify CaESS1 sequence with NdeI site |
| OW1355  | Reverse primer to amplify CaESS1 sequence with Sphi site |
| OW216   | Forward primer to amplify CaESS1 region with native stop codon (TAA) |
| OW1231  | Reverse primer to amplify CaESS1 region with native stop codon (TAA) |
| OW749   | Forward primer to amplify downstream portion of the linker swap |
| OW748   | Reverse primer to amplify upstream portion of the linker swap |
| OW751   | Forward primer to amplify downstream portion of the helix mutant |
| OW750   | Reverse primer to amplify upstream portion of the helix mutant |
| OW1224  | Forward primer to amplify upstream portion of the helix mutant/ linker swap |
| OW1233  | Reverse primer to amplify downstream portion of the helix mutant/linker swap |
| D1      | Forward primer to amplify ACT1 |
| D2      | Reverse primer to amplify ACT1 |
| D3      | Forward primer to amplify UR43 |
| D4      | Reverse primer to amplify UR43 |
| D6      | Forward primer to amplify APE2 |
| D7      | Reverse primer to amplify APE2 |
| D31     | Forward primer to amplify the sequence downstream of CaESS1 (+729 to +1265) with NotI ends |
| D32     | Forward primer to amplify CaESS1 |
| D33     | Reverse primer to amplify CaESS1 |
| UP043   | FGR46 GSP (detect reverse CUT) |
| UP044   | FGR46 GSP (detect coding region) |
| D52     | FGR46 rt-PCR (F) |
| D53     | FGR46 rt-PCR (R) |
| UP045   | snR3a GSP primer |
| UP046   | snR3a rt-PCR (F) |
| UP047   | snR3a rt-PCR (R) |
| UP051   | snR3a GSP primer |
| UP052   | snR3a rt-PCR (F) |
| UP053   | snR3a rt-PCR (R) |
| UP055   | snR3a GSP primer |
| UP056   | snR3a rt-PCR (F) |
| D111    | ACT1 GSP primer |

Lower case sequences added to obtain appropriate length. Bold indicates a mutation or restriction site.

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Table 5. Plasmids used in this study.

| Plasmid Name | SelectableMarker | Plasmid Content of Interest |
|--------------|------------------|-----------------------------|
| pDS426(pm)   | URA3             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) |
| pDS426(sw)   | URA3             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) |
| pDS413(pm)   | HIS3             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) |
| pDS413(sw)   | HIS3             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) |
| pDS(b)Leu    | LEU2             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × LEU2 |
| pDS(b)His    | HIS1             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × HIS1 |
| pDS(c)Leu    | LEU2             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × LEU2 × d.s.CaEss1(+572 to +1265) |
| pDS(c)His    | HIS1             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × HIS1 × d.s.CaEss1(+572 to +1265) |
| pDS(d)Leu    | LEU2             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × LEU2 × d.s.CaEss1(+572 to +1265) *no Ndel site in vector backbone |
| pDS(d)His    | HIS1             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × HIS1 × d.s.CaEss1(+572 to +1265) *no Ndel site in vector backbone |
| pDS(e)Leu    | LEU2             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × LEU2 × d.s.CaEss1(+572 to +1265) *Ndel site introduced at ATG start codon |
| pDS(e)His    | HIS1             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × HIS1 × d.s.CaEss1(+572 to +1265) *Ndel site introduced at ATG start codon |
| pDS(f)Leu    | LEU2             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × LEU2 × d.s.CaEss1(+572 to +1265) *Ndel site introduced at ATG start codon *Sphi site (TGC) introduced at TAA stop codon |
| pDS(g)Leu    | LEU2             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × LEU2 × d.s.CaEss1(+572 to +1265) *Ndel site introduced at ATG start codon *Sphi site (TGC) introduced at TAA stop codon |
| pDS(h)His    | HIS1             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × HIS1 × d.s.CaEss1(+572 to +1265) *no Ndel site at ATG start codon *Sphi site (TGC) introduced at TAA stop codon |
| pDS(h)Leu    | LEU2             | pCaEss1(-255 to 0) × terCaEss1(+535 to +735) × LEU2 × d.s.CaEss1(+572 to +1265) *no Ndel site at ATG start codon *Sphi site (TGC) introduced at TAA stop codon |

Growth assays

Overnight cultures of either S. cerevisiae or C. albicans were diluted to an OD_{600} of 0.1 and grown with shaking at 30°C until midlog phase. Cultures were brought to an OD_{600} of 0.5 either by dilution or concentration by centrifugation. For S. cerevisiae, a 1:5 dilution series of the strains were spotted onto plates containing selective media and incubated for two nights. For C. albicans strains, a 1:3 dilution series was used, with cells spotted on YPD plates and incubated overnight.

Filamentation assays

Overnight cultures of C. albicans were diluted to an OD_{600} of 0.25 (~3×10^{6} cells/ml). From dilution, 2 μl of culture was spotted onto Spider media [80] and serum-containing media (4% FBS and 2% agar). The plates were incubated at 37°C for 4 days prior to being photographed. To test germ tube formation, a suspension of 10^{2}–10^{6} cells/ml was made in liquid Spider medium [80], and incubated at 37°C for 2 hrs prior to microscopic observation.

Western blot analysis

Overnight cultures were diluted to an OD_{600} of 0.1, grown to an OD_{600} of 0.5 and harvested by centrifugation. Protein extracts were prepared using yeast protein lysis buffer (50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 1% Triton X and, 0.1% sodium deoxycholate) with proteinase inhibitor cocktail (ethanolic protease inhibitors with 0.25 M PMSF and 0.7 mg/ml pepstatin/aqueous protease inhibitors with 0.1 mg/ml leupeptin, 1 mg/ml soybean trypsin inhibitor, 0.1 mg/ml aprotinin in 10 mM Tris, pH7.5, 20 mM benzamidine, 10 mM sodium vanadate and, 500 mM sodium fluoride). A 15% polyacrylamide gel was run at 100 V for about 2.5 hrs and transferred to a PVDF Transfer Membrane (Millipore) overnight at 32 °C at 4 °C. A 1:500 dilution of rabbit anti-CaEss1 polyclonal antibodies (Applied Biosystems) was used as the primary antibody and a 1:25,000 dilution of horseradish peroxidase-linked donkey anti-rabbit IgG antibody (GE Healthcare) was used as the secondary antibody. Purified CaEss1 made in E. coli [50] was used to generate CaEss1 polyclonal antibodies at Applied Biosystems. Detection was done using the ECL-Plus Western Blotting Detection System (GE Healthcare) and exposure to X-ray film (Kodak).

High-throughput RNA sequencing

RNA and protein preparation. For the ts-readthrough mutant and control strain CaDS-C (Caess1Δ/Caess1^{TGC}) and CaDS-FC (Caess1Δ/Caess1^{TAC}) the following protocol was used. Cultures (10 ml) were grown overnight in selective media at 30°C, diluted 10-fold in YPD, and grown overnight again to an OD_{600} of 9–11 [81]. Cultures were diluted 30 times in pre-warmed (42°C), pre-shaken fresh YPD (1 ml in 29 ml YPD) [81], and cells were grown at 42°C with shaking. After 1 hr, 2 ml of culture was collected for protein preparation as above and the remaining culture diluted 2-fold in pre-warmed (42°C), pre-shaken YPD+20% FBS (20 ml culture in 20 ml YPD+20% FBS) for serum induction. After 2 hr incubation at 42°C, cultures were collected by centrifugation for protein preparation (2 ml) and...
RNA was extracted by the hot phenol method [82]. For all RNA extraction (10 ml). At extraction the cells were at an OD600 of 0.4. RNA was quantified using a Nanodrop Spectrophotometer and analyzed by Western blot analysis to confirm reduced amount before and after the temperature shift and serum induction were analyzed by Western blot analysis to confirm reduced amount of Eas1 protein at the restrictive temperature (42°C).

**Table 6. Strains used in this study.**

| Strain name       | Parent strain | Genotype                                                                 | Source                        |
|-------------------|---------------|--------------------------------------------------------------------------|------------------------------|
| SC5314            | clinical isolate | Wild-type                                                                | Fonzi and Irwin (1993)       |
| CAI4              | SC5314        | ura3Δ: imm434/ura3Δ: imm434                                               | Fonzi and Irwin (1993)       |
| CaGD1             | CAI4 (*)      | ESS1/ess1Δ: hisG-URA3-hisG ura3Δ: imm434/ura3Δ: imm434                   | Devasaahayam et al., 2002   |
| CaGD2             | CAI4          | ESS1/ess1Δ: hisG ura3Δ: imm434                                            | Devasaahayam et al., 2002   |
| R6                | CaGD2         | ESS1/ESS1: hisG-URA3-hisG ura3Δ: imm434/ura3Δ: imm434                   | Devasaahayam & Hanes, unpublished |
| R6*               | R6            | ESS1/ESS1: hisG ura3Δ: imm434                                            | this study                   |
| CaGD2*+pLUBP (−/+ URA3 at native) | CaGD2 | ESS1/ess1Δ: hisG URA3/ura3Δ: imm434                                       | this study                   |
| R6*+pLUBP (−/+ URA3 at native) | R6*        | ESS1/ESS1: hisG URA3/ura3Δ: imm434                                       | this study                   |
| CaGD2*+Clp10 (−/+ URA3 at RPS1) | CaGD2 | ESS1/ess1Δ: hisG ura3Δ: imm434/ura3Δ: imm434 rps1Δ: URA3/RPS1             | this study                   |
| R6*+Clp10 (+/+ URA3 at RPS1) | R6*        | ESS1/ESS1: hisG ura3Δ: imm434                                            | this study                   |
| CAI4*+pLUBP URA3 at native | CAI4 (*) | URA3/ura3Δ: imm434                                                       | this study                   |
| CAI4*+Clp10 URA3 at RPS1 | CAI4 (*) | ura3Δ: imm434/ura3Δ: imm434 rps1Δ: URA3/RPS1                             | this study                   |
| CAI12             | CAI4          | URA3/ura3Δ: imm434                                                       | Porta et al., 1999          |
| CaDS-B0.5         | SN87          | ESS1/ESS1: C.m.LEU2 leu2Δ/leu2Δ his1Δ/ his1Δ URA3/ura3Δ: imm434          | this study                   |
| CaDS-B5           | CaDS-B0.5     | ESS1/ES1: C.m.HIS1/ESS1: C.m. LEU2 leu2Δ/leu2Δ his1Δ/ his1Δ URA3/ura3Δ: imm434 | this study                   |
| CaDS-B5.5         | CaDS-B0.5     | ess1Δ: C.m.HIS1/ESS1: C.m. LEU2 leu2Δ/leu2Δ his1Δ/ his1Δ URA3/ura3Δ: imm434 | this study                   |
| CaDS-B7-2.3       | SN87          | ess1Δ: C.m.HIS1/ESS1: C.m. LEU2 leu2Δ/leu2Δ his1Δ/ his1Δ URA3/ura3Δ: imm434 | this study                   |
| CaDS-C            | CaDS-B7-2.3   | ess1Δ: C.m.HIS1/ess1Δ: leu2Δ/leu2Δ his1Δ/ his1Δ URA3/ura3Δ: imm434        | this study                   |
| CaDS-FC           | CaDS-C        | ess1Δ: C.m.HIS1/ess1Δ: leu2Δ/leu2Δ his1Δ/ his1Δ URA3/ura3Δ: imm434        | this study                   |
| CaDS-E            | SN87          | ESS1/ess1Δ: C.m. LEU2 leu2Δ/leu2Δ his1Δ/ his1Δ URA3/ura3Δ: imm434        | this study                   |
| CaDS-E5           | CaDS-E        | ess1Δ: C.m.HIS1/ess1Δ: leu2Δ/leu2Δ his1Δ/ his1Δ URA3/ura3Δ: imm434        | this study                   |
| CaDS-E5.10        | CaDS-E        | ess1Δ: C.m.HIS1/ess1Δ: leu2Δ/leu2Δ his1Δ/ his1Δ URA3/ura3Δ: imm434        | this study                   |
| CaDS-S1B-2        | SN87          | ESS1-5’ape2Δ: C.m.HIS1/ESS1-5’ape2Δ: C.m. LEU2 leu2Δ/leu2Δ his1Δ/ his1Δ URA3/ura3Δ: imm434 | this study                   |

(C.d.) Candida dubliniensis; (C.m.) Candida maltosa; (5’) promoter region truncated; (hm) helix mutant. Abbreviations: (p) promoter region; (ter) termination region; (sw) linker swap; (hm) helix mutant; (d.s.) downstream sequence. (*) may have acquired a mutation that affects filamentation as per this study.

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Library preparation and high-throughput sequencing. RNA samples (1 μg in 10 μl of RNase free water) were sent for library preparation and sequencing to off-site facilities (Weill Cornell Medical College, NY and Centrillion Biosciences, CA). At Cornell, from 1 μg of purified RNA, ribosomal RNA was removed using Ribo-Zero rRNA Removal Kit (Epicenter). Illumina-compatible, barcoded, strand-specific, cDNA libraries were prepared using ScriptSeq mRNA-Seq Library Preparation Kit and RNA-Seq Barcode Primers (Epicenter). Illumina high-throughput sequencing was performed on the libraries using the HiSeq2000 sequencer running at 58 cycles (per lane) resulting in one-end read lengths of about 51 bp. At Centrillion, a similar approach was taken to prepare Illumina-compatible, barcoded, strand-specific, cDNA libraries with the exceptions of using the RiboMinus Eukaryote Kit (Invitrogen) for removal of ribosomal RNA and the ScriptSeq V2 RNA-Seq...
Library Preparation Kit (Epitecter). The ScriptSeq V2 Kit is an improved version of the ScriptSeq Kit and uses the same basic principal for library construction. Here the HiSeq2000 sequencer was run at 50 x 2 cycles (per lane) resulting paired-end read lengths of 100 bp.

**RNA sequence analysis**

The raw FastQ data files were aligned to the *C. albicans* genome (Assembly 21) [83] obtained from Candida Genome Database (http://www.candidagenome.org/) using Bowtie/TopHat short sequence read alignment software programs [84,85]. Each strain resulted in a large number of reads mapped to the genome (Table 2). The output files (in SAM format) were converted to BAM files using SAM Tools [86]. The BAM files were visually analyzed on Integrative Genomics Viewer (http://www.broadinstitute.org/igv/) and/or GenomeView (http://genomeweb.org/). The files were quantitatively analyzed using the Galaxy server (https://main.g2.bx.psu.edu/), an open access platform for high-throughput data analysis. For example, the SAM or BAM files were used as Cuffdiff (a part of the open-source Cufflinks software package) [85] input files to identify differential expression of transcripts genome-wide among test strains and controls.

**Quantitative reverse transcription real-time PCR**

Overnight cultures were diluted to an OD$_{600}$ of 0.5 and grown to an OD$_{600}$ of 0.8–1.2 and harvested. RNA was extracted using the hot phenol method [82] and subjected to DNase digestion (Epitecter) for 45 min at 37°C. From 1 μg of RNA, cDNA was synthesized using the first Strand cDNA Synthesis Kit for Real-Time PCR/USB using mixture of oligo dT and random hexamers. In some sets of cDNA experiments cDNA was synthesized using individual cDNA synthesis reagents purchased from USB/Affimix. Quantitative real-time PCR was performed in the ABI2000 RT-PCR machine using the HotStart-IT SYBR Green qPCR Master Mix (USB). In some sets of experiments real-time PCR was carried out using the Fermentus SYBR Green Master Mix (Life Sciences). All reactions were normalized against the same internal control gene, ACT1, qRT-PCR calculations were done as per Yu et al., [87] where the quantitative real-time PCR cycle numbers relative to ACT1 were summed for each biological replicate and normalized to the summed average of all samples within a given experiment. The re-normalized values were then used for average and standard deviation calculations. This method corrects for trial-to-trial variability. To calculate the fold difference between samples, the $ACT1_{	ext{normalized (ACt)}}$ values for the FC (WT/-) and C (ts/-) data sets were normalized against the WT/ WT (B5) data to obtain $\Delta$ACt, and the following calculation was performed: fold difference $= 2^{-\Delta ACt}$. The values obtained were averaged, and a standard deviation calculated.

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**Author Contributions**

Conceived and designed the experiments: DS DA RM JTW VC SDH. Performed the experiments: DS DA. Analyzed the data: DS DA RM JTW VC SDH. Contributed reagents/materials/analysis tools: DS DA RM JTW VC SDH. Wrote the paper: DS SDH.

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