Proper Sterol Distribution Is Required for Candida albicans Hyphal Formation and Virulence

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ABSTRACT Candida albicans is an opportunistic fungus responsible for the majority of systemic fungal infections. Multiple factors contribute to C. albicans pathogenicity. C. albicans strains lacking CaArv1 are avirulent. Arv1 has a conserved Arv1 homology domain (AHD) that has a zinc-binding domain containing two cysteine clusters. Here, we explored the role of the CaAHD and zinc-binding motif in CaArv1-dependent virulence. Overall, we found that the CaAHD was necessary but not sufficient for cells to be virulent, whereas the zinc-binding domain was essential, as Caarv1/Caarv1 cells expressing the full-length zinc-binding domain mutants, Caarv1C3S and Caarv1C28S, were avirulent. Phenotypically, we found a direct correlation between the avirulence of Caarv1/Caarv1, Caarv1AHD, Caarv1C3S, and Caarv1C28S cells and defects in bud site selection, septa formation and localization, and hyphal formation and elongation. Importantly, all avirulent mutant strains lacked the ability to maintain proper sterol distribution. Overall, our results have established the importance of the AHD and zinc-binding domain in fungal invasion, and have correlated an avirulent phenotype with the inability to maintain proper sterol distribution.

Saccharomyces cerevisiae ARV1 (Are1 Are2 required for viability) was identified in a genetic screen looking for recessive alleles toxic to are1 are2 cells (Tinkelenberg et al. 2000). The S. cerevisiae ARE1 and ARE2 genes are required for yeast sterol esterification (Yang et al. 1996). Cells lacking both are viable, but are unable to esterify sterols, thus accumulate free sterol, while are1 are2 arev1 cells are not (Zweytick et al. 2000). Fungi that express Arv1 include C. albicans and C. glabrata (Gallo-Ebert et al. 2012; Tinkelenberg et al. 2000). All fungal Arv1 proteins have a conserved amino-terminal Arv1 homology domain (AHD) that contains a consensus zinc-binding motif [C–xx–C–(~20)–CxxC] (Figure 1) (Fores et al. 2006). The topology of the S. cerevisiae Arv1 has been solved. It has three endoplasmic reticular transmembrane-spanning regions, a cytoplasmic-facing AHD, and a single large luminal loop region (Georgiev et al. 2013; Villasmil and Nickels 2011). arv1 cells are hypersusceptible to the ergosterol-binding agent nystatin, suggesting a mislocalization of sterol to the plasma membrane (Tinkelenberg et al. 2000). Cells lacking Arv1 accumulate several unknown sterol intermediates, suggesting these cells have defects in sterol synthesis (Kajiwara et al. 2008), and they harbor lipid distribution defects, as they cannot polarize phosphatidylinositol 4,5 phosphate (PIP2) during yeast mating (Fei et al. 2008; Villasmil et al. 2011). Mutant cells also have defects in organelle lipid morphology and homeostasis (Georgiev et al. 2013; Schechtmans et al. 2011), and they are highly sensitive to fatty acid supplementation (Ruggles et al. 2014).

Candida albicans and Candida glabrata are pathogenic fungi responsible for the majority of systemic candidiasis cases (Pfaller 1996; Segal 2005; Spellberg 2008). Both are becoming resistant to multiple antifungal drugs, especially the azole class of drugs, and this contributes to clinical resistance (Cowen 2008; Perlin 2014; Rodrigues et al. 2014; Shields et al. 2015). Although the sterol biosynthesis pathway has become a “hot spot” for acquiring azole resistance (Asai et al. 1999; Denning et al. 1997; Sanglard et al. 1998; Vermitsky and Edlind 2004; Xu et al. 2008), it still may be advantageous to target factors involved in maintaining sterol homeostasis. (Borjihan et al. 2009; Henneberry and Sturley 2005; Simova et al. 2013; Zhang and Rao 2010). Our hypothesis is that disrupting cellular sterol distribution will lead to avirulence. Thus, cell factors regulating this process represent novel drug targets. We believe that Arv1 may represent such a target.

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arv1 cells are highly susceptible to the desippeptide phosphatidylserine binding agent papuamide-B, indicating elevated levels in the plasma membrane (Georgiev et al. 2013). Thus, there is strong evidence that Arv1 regulates lipid distribution in S. cerevisiae.

C. albicans Caaarv1/Caaarv1 cells are hypersusceptible to the polyenes, nystatin and amphotericin B, itraconazole, and lovastatin (Gallo-Ebert et al. 2012). As in the case of S. cerevisiae arv1 cells, the growth defects observed in the presence of the polyenes and papuamide-B suggest that lipid distribution is perturbed at the plasma membrane.

We have previously found, using a disseminated candidiasis mouse model, that Caaarv1/Caaarv1 cells were avirulent (Gallo-Ebert et al. 2012). In cell culture, Caaarv1/Caaarv1 cells had defects in hyphae formation and elongation (Gallo-Ebert et al. 2012), and defects in sterol distribution along the growing hyphae (Gallo-Ebert et al. 2012), suggesting that CaArv1 regulates sterol distribution, and proper sterol distribution is required for virulence. Uncovering the motifs required for Arv1 function will help to understand the molecular basis for Arv1-driven virulence, while further underscoring the importance of maintaining proper lipid distribution during fungal infection.

Here, we explored the importance of the CaAHD and zinc-binding motifs in CaArv1-dependent virulence. We found that the CaAHD alone cannot replace full-length Arv1 in conferring virulence, while also demonstrating that Cys3 and Cys28 within the zinc-binding motif are essential, as cells expressing full-length CaAHD alone, Caarv1/Caarv1 display hyphal formation and elongation defects, as well as sterol distribution defects along the growing hypha. Overall, there was a direct correlation between cells having the ability to maintain proper sterol distribution and virulence, validating our hypothesis that maintaining proper sterol localization is critical for fungal pathogenicity.

MATERIALS AND METHODS

Strain and plasmid construction

Yeast transformations were performed using the Frozen EZ Yeast Transformation II Kit (Zymo Research). All C. albicans strains were generated using BWP17 (Table 1). The PCR-based gene disruption method was used for disruption of CaARV1 (Norice et al. 2007). Primers used for cloning are listed in Table 2. Primers (CaARV1-5DR and CaARV1-3DR) were constructed containing 20 bp homologous to the disruption plasmid, pGEM-URA3 and pRS-ARG4, respectively. Disruption of the endogenous CaARV1 allele was verified by PCR (primers CaARV1-CON5F and CaARV1-CONF3R). All expression plasmids contained 500 bp of the endogenous CaARV1 promoter, and 500 bp of the CaARV1 terminator. CaARV1 (CaARV1-5 COMP and CaARV1-3 COMP), CaARV1(CaARV1-5 COMP and CaARV1-3 COMP), and CaARV1(AHD (CaARV1-AHD-BamHI and CaARV1-AHD-Sall) alleles were generated by PCR. To integrate CaARV1 alleles into a Caaarv1/Caaarv1 homozygous deletion strain, each individual allele was PCR-amplified containing NotI sites. Thus, all Caaarv1/Caaarv1 strains expressing various arv1 alleles are heterozygous for each allele. PCR fragments were cloned into the disruption plasmid, pDDB78-HIS1 (Spreghini et al. 2003), which was linearized with NruI, and integrated into the Caaarv1/Caaarv1 homozygous deletion strain at the HIS1 locus. Integration was verified by PCR amplification of the HIS1 locus (CaHIS-ARV1-DIAG5F, CaHIS-ARV1-DIAG3R, CaHIS-PGEM-DIAG3R, and CaHIS-pDDB78-HIS1). Full-length CaARV1 was used to construct site-directed mutants (CaARV1-C3A-SDM5F, CaARV1-C3A-SDM3R, CaARV1-C28A-SDM5F, CaARV1-C28A-SDM3R, and CaARV1-C28S-SDM5F, CaARV1-C28A-SDM5F). Heterozygous Caaarv1/Caaarv1 transformants were selected on synthetic minimal medium lacking uracil, arginine, and histidine, thus obtaining URA3+, ARG4+, and HIS1+ transformants. Integration was verified by PCR. All strains were integrated with all selectable markers (URA3, ARG4, HIS4) to eliminate auxotrophic-specific pleiotropic effects. All mutant plasmid constructs were sequenced to verify the presence of individual mutations. qRT-PCR indicated that there were no copy differences in the expression of the alleles. Point mutations were generated using the QuickChange Site Directed Mutagenesis Kit (Stratagene) and pDDB78-HIS1-CaARV1 (pHIS1) as a template. The endogenous CaARV1 promoter drove expression of all constructs.

To construct Caaarv1/Caaarv1 cells expressing CgARV1, the full-length coding sequence of CgARV1 was subcloned into pDDB78-HIS1 containing 500 bp of the CaARV1 promoter, full length CgARV1 (1000 bp), and 500 bp of the CaARV1 terminator. Caaarv1/Caaarv1 cells expressing AHD were generated by integrating pDDB78-HIS1 containing the CaARV1 AHD domain.

Protein isolation and western analysis

Protein extraction was performed as described previously (Villasmiil and Nickels 2011). Protein levels were determined using cell lysates and the Bradford assay system (Bio-Rad). Proteins were visualized using immunoblotting and chemoluminescence as described previously (Villasmiil and Nickels 2011). Rabbit anti-yeast Arv1 polyclonal antibodies were generated by Lampire Biological Products (Pipersville, PA), and were used at a 1:500 dilution.

Sterol extraction and analysis

Single colonies of Candida albicans strains were grown for 18 hr in YEPD at 37°C, 200 rpm; ~15 ml of culture was harvested and cells were washed twice with ddH2O. Cells were then resuspended in 1 ml ddH2O and split equally into two tubes, one sample for the determination of the dry weight of cells, the other for sterol extraction.

Nonsaponifiable lipids were prepared and extracted as reported previously (Kelly et al. 1995). An internal standard of 10 μg of cholesterol was added prior to extraction with hexane. Samples were dried in a vacuum centrifuge (Heto), and were derivatized by the addition of 100 ml 90% N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)/10% trimethylsilyl (TMS) (Sigma) and 200 ml anhydrous pyridine (Sigma), and heating for 2 hr at 80°C. TMS-derivatized sterols were analyzed and identified using GC/MS (Agilent 5975C Inert XL GC/MSD) with reference to retention times and fragmentation spectra for known standards. GC/MS data files were analyzed using Agilent software (MSD).
Enhanced ChemStation, Agilent Technologies, Stockport, UK) to determine integrated peak areas, and enable calculation of the percentage of total sterols and the amount of sterol/dry weight of cells.

**Disseminated candidiasis studies**

Female BALB/c mice (Jackson Labs) aged 6–8 wk, weighing ~18–22 g, were housed in groups of as many as four animals, and were supplied food and water *ad libitum*; 8–10 mice were used for each strain. *C. albicans* strains were grown overnight in YEPD medium (1% yeast extract, 2% bactopeptone, and 2% dextrose) at 30°, harvested by centrifugation, washed twice with 1× phosphate-buffered saline (PBS), counted by hemocytometry, and resuspended in 1 ml of water; 200 μl of each strain were injected via the tail vein with 200 cells/ml of *C. albicans* in 1× PBS. Infected animals were monitored daily for 30 d postinfection, and were considered moribund when they could no longer reach food or water. Moribund animals and mice surviving to the end of the study were killed by CO₂ asphyxiation, and survival times were recorded. All animals were housed at Temple University—an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) accredited facility. The Temple University Institutional Animal Care and Use Committee (IACUC) approved the protocol.

For organ fungal load determination, mice were injected via the tail vein with 200 μl of 5 × 10⁶ cells/ml of *C. albicans* in 1× PBS. Infected animals were monitored daily for 30 d postinfection, and were considered moribund when they could no longer reach food or water. Moribund animals and mice surviving to the end of the study were killed by CO₂ asphyxiation, and survival times were recorded. All animals were housed at Temple University—an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) accredited facility. The Temple University Institutional Animal Care and Use Committee (IACUC) approved the protocol.

**Determination of organ fungal load**

Mice infected with *C. albicans* were killed 48 hr postinfection, and target organs (kidney, spleen, and liver) were removed aseptically and homogenized in 4 ml of 1× PBS. Fungal load was determined by making 10-fold serial dilutions in 1× PBS, and plating 40 μl on YEPD plates containing 34 μg/ml chloramphenicol. Plates were incubated at 30° for 24 hr. Total CFUs were determined, and counts were expressed as the log₁₀ CFU/organ weight in grams; 8–10 organs were combined and analyzed.

**Chitin staining and fluorescence microscopy**

Hyphal formation was induced at 37° for 3 hr in 10% fetal bovine serum (FBS). Cells were fixed with 2% paraformaldehyde for 10 min at room temperature, followed by gentle washing with PBS. Cells were stored at 4° until microscopic analysis. Bud scars were visualized by calcofluor white staining (Sigma-Aldrich, 50–100 μg/ml), with an incubation of 2–5 min at room temperature. Microscopy was performed immediately with 100× magnifications using a Leica fluorescence microscope with an attached camera. At least 300 cells were examined, and the data are the average of five independent experiments.

**Filipin staining and fluorescence microscopy**

Unesterified sterol was visualized using filipin staining. One milliliter of 37.5% formaldehyde was added to 9 ml of cell culture grown to a density of 0.7 OD₅₅₀ U/ml. After 10 min of mixing at 23°, fixed cells were centrifuged, and the pellet was washed twice with 10 ml distilled water. Washed cells were resuspended in 1 ml of water; 200 μl was mixed with 4 μl of freshly made 5 mg/ml filipin complex in ethanol (Sigma-Aldrich, St. Louis, MO). After incubating in the dark for 15 min, cells were spotted directly onto slides, and filipin fluorescence was observed with a UV filter set using neutral density filters. For all fluorescence microscopy experiments, samples were mounted on microscope slides, sealed under coverslips with nail polish, and imaged on a Leica fluorescence microscope with an attached camera. Three hundred cells were counted for each strain, and the data are the average of five independent experiments.

**Data availability**

Strains and all reagents are available upon request.

**RESULTS**

**Caarv₁AHD, Caarv₁C₃₅, and Caarv₁C₂₈₅ strains are avirulent**

Expressing the ScAHD alone can restore mating to *Caarv₁* cells (Villasmil et al. 2011), suggesting it can substitute for full-length ScArv1 function under some circumstances. To determine if the AHD could substitute for full-length ScArv1 in conferring virulence, a *Caarv₁/Caarv₁* strain was generated expressing a single *Caarv₁AHD* allele, and it was tested using a murine model of disseminated candidiasis. The percentage survival of *Caarv₁AHD*-infected mice was compared to those injected with CaARV1/CaARV1, Caarv₁/CaARV1, and Caarv₁/Caarv₁ cells.
Inmunoblot analysis showed that the Caarv<sup>1AHD</sup> strain expressed AHD at a level 2.5-fold higher than full-length CaArv1 (Figure 2, A and B). qRT-PCR indicated there were no differences in copy numbers (not shown).

Mice injected with CaARV1/CaArv1 cells were dead by d 18, with 50% lost by d 6 (Figure 3A, filled circles), and 50% of mice injected with CaARV1/CaArv1 cells were dead by d 5 (Figure 3A, open boxes) \((P < 0.0001)\) (Table 3). Twenty percent of the remaining mice survived from d 16 to the end of the study, while 100% of mice injected with CaArv1/CaArv1 cells survived until the study was terminated at 30 d (Figure 3A, filled squares) \((P < 0.0001)\). These results are in good agreement with previous work (Gallo-Ebert et al. 2012). Mice injected with CaArv1<sup>1AHD</sup> cells also survived the entire length of the study (Figure 3A, open triangles) \((P < 0.0001)\).

Next, we tested the role of the AHD zinc-binding domain in virulence. In this case, amino acids were changed in full-length CaArv1. Cysteines at positions Cys3 (Caarv<sup>1C3S</sup>) and Cys28 (Caarv<sup>1C28S</sup>) were mutated in the first and second cysteine clusters of the zinc-binding domain (Figure 1, underline). We substituted each Cys with Ser in order to retain tertiary structure (Botello-Morte et al. 2016; Stachowiak et al. 2009). Immunoblotting analysis showed that CaArv1, CaArv1<sup>C3S</sup>, and CaArv1<sup>C28S</sup> were expressed equally (Figure 2, A and B). Again, qRT-PCR indicated that the copy number of each allele was similar (not shown). Mice injected with either Caarv1<sup>1C3S</sup> (Figure 3B, open circles) or Caarv1<sup>1C28S</sup> (Figure 3B, open triangles) cells survived for the length of the study \((P < 0.0001; P < 0.0003)\).

Finally, we explored the conservation of Arv1 function by integrating a single CgArv1 allele into Caarv1/CaArv1 cells and testing for virulence. Fifty percent of mice injected with CgArv1<sup>Cgaarv1</sup> cells died between d 7 and 8 (Figure 3B, open pyramids) \((P < 0.05)\). The remaining mice were dead by d 10.

Our results together indicated that the AHD alone does not possess the same function as full-length CaArv1. They also show that zinc-binding domain function is needed to confer virulence, while indicating a degree of conservation between CaArv1 and CgArv1 alleles.

**Abnormal organ fungal loads are seen in mice injected with Caarv1<sup>1C3S</sup>, Caarv1<sup>1C28S</sup>, and Caarv1<sup>CgArv1</sup> cells**

Multiple tissue failure contributes to the mortality associated with disseminated candidiasis, as organ colonization and invasion is normally seen during an invasive infection (de Repentigny 2004). Thus, fungal loads were determined in the kidney, liver, and spleen, in order to determine if there was any correlation between an increase in organ colonization and virulence.

All organ fungal load levels were similar in CaArv1/CaArv1- and Caarv1/CaArv1-injected mice (Figure 4). On the other hand, mice injected with Caarv1/CaArv1 cells had reduced fungal loads in the kidney, spleen, and liver (Figure 4, Caarv1/CaArv1 vs. CaArv1/CaArv1). These results are in good agreement with previous results (Galbo-Ebert et al. 2012). The organ fungal loads of Caarv1<sup>1AHD</sup>-injected mice were similar to that seen for CaArv1/CaArv1 and Caarv1/CaArv1 cells, an interesting observation in light of the avirulence of this strain. Mice injected with Caarv1<sup>1C3S</sup> and Caarv1<sup>1C28S</sup> cells had reduced fungal loads in all organs. The reduction in fungal load levels in these mutants directly correlates well with the degree of virulence. Unexpectedly, we found that mice injected with Caarv1<sup>Cgaarv1</sup> cells had reduced fungal load levels (Figure 4).

Overall, our results showed that mice injected with Caarv1<sup>1C3S</sup> and Caarv1<sup>1C28S</sup> cells had lower fungal loads, and this correlated with increased survival. On the other hand, mice injected with Caarv1<sup>1AHD</sup> cells, which were avirulent, had normal fungal load levels. Finally, Caarv1<sup>Cgaarv1</sup> cells displayed a higher degree of virulence than did all other tested cells, even though the fungal load levels of mice injected with these cells were drastically lower.

**Avirulent mutant cells expressing Caarv1<sup>1AHD</sup>, Caarv1<sup>1C3S</sup>, and Caarv1<sup>1C28S</sup> have altered sterol levels**

There is strong evidence that Arv1 regulates sterol homeostasis and localization (Galbo-Ebert et al. 2012; Georgiev et al. 2013; Ruggles et al. 2014; Swain et al. 2002b; Tinkenberg et al. 2000). Caarv1 cells

| Primer Name | Sequence |
|-------------|----------|
| CaArv1-5DR (CaArv1 deletion) | 5′-CTGCTCTGATACACTGAGGCATTCAACGCGCATTTACATTGAGG<br>AAGATACCGGGAATGACACCATCCTGTACACTTCTTTTCACATCAGCAGT-3′ |
| CaArv1-3DR (CaArv1 deletion) | 5′-AATTGACAACTAATACGAAATCAGATGTAGTTTACGTTAGG<br>CAGGCGCAATTTATGGAATTTGGAATGCGGAAATG-3′ |
| CaArv1-CON5F (CaArv1 deletion verification) | 5′-GGCAACCACTTACGAAATCCG<br>CAGGCGCAATTTATGGAATTTGGAATGCGGAAATG-3′ |
| CaArv1-CON3R (CaArv1 deletion verification) | 5′-CCTTGGAGCATTGAAAACG-3′ |
| CgArv1-CONF2 (CgArv1 allele integration verification) | 5′-CAATATGGCTTCTCTTCT-3′ |
| CgArv1-CONIR (CgArv1 allele integration verification) | 5′-GCCCATTGAGGTGAATCT-3′ |
| CaArv1-5 COMP (CaArv1 allele generation) | 5′-CAAGAATTTGCCATCCCAAA-3′ |
| CaArv1-5 COMP (CaArv1 allele generation) | 5′-ACACCAAATGATTTGCA-3′ |
| CaArv1-AHD-BamHl (CaAHD allele generation) | 5′-TTACTGATTATTGCCAACCT-3′ |
| CaArv1-AHD-Sall (CaAHD allele generation) | 5′-GGGTGAACTTTTAAATGATTTGGAATG-3′ |
| CaArv1-C3S-SDMSF (C3S allele generation) | 5′-CCATTTTTTAAATGATTTGGAATGATTTT-3′ |
| CaArv1-C3S-SDM3R (C3S allele generation) | 5′-TTACCATTTTCAATGATTTGGAATGATTTT-3′ |
| CaHIS-ARV1-DIAG3R (HIS1 integration verification) | 5′-GTTGGTGGTGCACAGAC-3′ |
| CaHIS-ARV1-DIAG3R (HIS1 integration verification) | 5′-GTGACAACTCGTATGCTCC-3′ |
| CaARV1-C28S-SDM5F (C28S allele generation) | 5′-TATATCAAACTAATGTAAGTCGCCAATGTAATAAAA-3′ |
| CaARV1-C28S-SDM3R (C28S allele generation) | 5′-TTATATCAAACTAATGTAAGTCGCCAATGTAATAAAA-3′ |
| CaHIS-ARV1 DIAG5F (HIS1 integration verification) | 5′-GGTTGGTGGTCACAGAC-3′ |
| CaHIS-ARV1 DIAG5R (HIS1 integration verification) | 5′-GGTGACAACTCGTATGCTCC-3′ |
| CaHIS-PGEM-DIAG-3R (HIS1 integration verification) | 5′-CCGCGGCGCATGCC-3′ |
| CaHIS-PDDB78-DIAG-3R (HIS1 integration verification) | 5′-TCAAGGTCGACGGTATCG-3′ |
accumulate unknown sterols, and a direct correlation exists between accumulation of these intermediates and Scarv1 phenotypes (Georgiev et al. 2013b; Swain et al. 2002b; Tinkelenberg et al. 2000). Moreover, these cells display sterol distribution defects. Caarv1/Caarv1 cells are avirulent (Gallo-Ebert et al. 2012), and have defects in sterol distribution. To see if there was a correlation between defects in sterol composition and avirulence, sterol intermediates were quantified and their levels were calculated as the percentage of sterol intermediate/total sterol (Table 4).

Interestingly, the sterol compositions of CaARV1/CaARV1 and Caarv1/Caarv1 cells were different (Table 4, WT vs. hetero). Heterozygous cells had a higher percentage of ergosterol (130%), and decreased percentages of zymosterol (28%), episterol (25%), fecosterol (23%), ergosta-5,7,24(28)-tri-enol (23%), and lanosterol (23%) compared to CaARV1/CaARV1 cells (Table 4). Caarv1/Caarv1 cells had a higher percentage of ergosta 5,7 dienol (300%), and a lower percentage of zymosterol (26%) compared to CaARV1/CaARV1 cells.

To next examine if the AHD, Cys3, and Cys28 were required for maintaining normal sterol composition, sterol content was determined in cells expressing Caarv1AHD, Caarv1C3S, or Caarv1C28S alleles. Sterol intermediates levels were compared to Caarv1/Caarv1 cells. Caarv1AHD cells had a higher percentage of ergosta 5,7 dienol (246%) and a lower percentage of 4,4-dimethylzymosterol (10%), whereas both Caarv1C3S and Caarv1C28S cells had higher percentages of fecosterol (415%), ergosta-5,7,24(28)-tri-enol (630%), ergosta 5,7 dienol (454%), and lanosterol (242%). The sterol composition of Caarv1C28S cells was similar to that of Caarv1/Caarv1 cells.

In looking at the data as a whole, it is interesting that all avirulent strains accumulated the same sterol intermediate, ergosta 5,7 dienol: [Caarv1/Carv1 (300%), Caarv1AHD (246%), Caarv1C3S (630%), and Caarv1C28S (630%)].

**Proper hyphal formation is delayed in avirulent strains**

Hyphal formation and subsequent elongation are necessary for strains to be virulent (Lu et al. 2014). In order to understand the molecular basis underlying avirulence, hyphal initiation and formation were visualized in cell culture using fluorescence microscopy (Figure 5A). Cells were visualized at 3 hr after growth in invasive medium.

Interestingly, Caarv1/Caarv1 cells did have a reduction in the number of cells forming hyphae compared to CaARV1/CaARV1 cells (Figure 5B, P < 0.001). The percentages of Caarv1/Caarv1, Caarv1AHD, Caarv1C3S, and Caarv1C28S cells forming hyphae were also significantly reduced (Figure 5B, P < 0.001; P < 0.001; P < 0.001, respectively). Caarv1/Caarv1 cells expressing CaARV1 did not display a reduction in hyphal formation.

**Avirulent cells have defects in bud site selection and septa formation**

Bud site selection along the mother cell periphery dictates where hyphal formation will initiate (Lu et al. 2014). The initial step of hyphal formation is delayed in avirulent strains.
biogenesis is the formation of the germ tube, which emerges in a predominately nonaxial position (bipolar or random). Hyphal branches then emerge adjacent to locations of hyphal septa, on the mother (proximal) side (Gow and Hube 2012; Hausauer et al. 2005). To further our understanding of why mutant cells were delayed in hyphal formation, we visualized septa number and location, and the positioning of bud sites using calcofluor white and fluorescence microscopy. Chitin localization was visualized in vitro after cells were grown in hyphae-inducing medium for 3 hr.

Examples of septa (Figure 6A, arrows) and chitin bud site staining (Figure 6B, asterisk) are shown posthyphal initiation for CaARV1/CaARV1 and Caarv1/Caarv1 cells, respectively. When examined, CaARV1/CaARV1 cells had ≥ 2 septa (Figure 6A, arrows) along a single hypha; ~85% of CaARV1/CaARV1 cells initiated hypha from a single mother–daughter chitin bud site at 3 hr postinitiation (Figure 6A, asterisk). The number of Caarv1/CaARV1 cells having ≥ 2 septa was reduced to ~30% of that seen in CaARV1/CaARV1 cells (Figure 6C); however, Caarv1/CaARV1 cells were normal for septa formation, and for the number and positioning of chitin bud sites. Caarv1/Caarv1 mutants had a reduction in the numbers of hyphae formed compared to CaARV1/CaARV1 cells (Figure 6C, ~65%, \( P < 0.001 \)). These mutants had constrictions along the germ tube, which lacked chitin staining (Figure 6B, hash sign), and hyphal initiation was initiated from a single bud site. Another interesting phenotype displayed by Caarv1/Caarv1 cells was that they had a second chitin bud site that was the initiating point for another germ tube (Figure 6B, asterisk). A high percentage of Caarv1\(^{AHD} \) (~95%), Caarv1\(^{C3S} \) (90%), and Caarv1\(^{C28S} \) (85%) cells had only a single chitin bud site that remained at the initial mother–daughter neck (Figure 6C, black bars), and ~75% of Caarv1\(^{CgARV1} \) cells had a single chitin bud site and ≥ 2 septa along a single hypha (Figure 6C, black bars).

**Avirulence correlates directly with defects in ergosterol distribution**

The loss of *S. cerevisiae* Arv1 causes sterol distribution defects (Georgiev et al. 2013; Villasmil et al. 2011). There is a direct correlation between the degree of sterol defects and a reduction in mating efficiency (Villasmil et al. 2011). Sterol distribution defects are also seen in Caarv1/Caarv1 cells, and severity correlates directly with loss of hyphal formation and the degree of avirulence (Galloy-Ebert et al. 2012). Thus, there is a relationship between loss of Arv1 function, defects in sterol distribution, and signaling-dependent polarized growth.

To see if there was a correlation between lack of sterol localization and avirulence, the localization of cellular sterol was visualized using filipin staining and fluorescence microscopy. Qualitatively, we found that all cells took up the same level of filipin, so we reasoned that any defects observed would not be due to lack of dye internalization.

**Figure 4** Organ fungal load analysis indicates differences between strains. Mice were injected with 10^6 cells/ml. Organs were harvested 2 d post injection. Each organ was homogenized and C. albicans CFUs were determined by plating homogenates onto YEPD plates containing chloramphenicol. Plates were incubated at 30° for 24 hr. Total CFUs were determined and counts were expressed as the log10 CFU/organ weight in grams. The values are the average values obtained from 8 to 10 combined organs from each strain. * *P < 0.001.

CaARV1/CaARV1 and Caarv1/CaARV1 cells had a similar percentage of cells having normal distribution (Figure 7), localizing the majority of their sterol to the growing hyphal tip (Figure 8, arrows). Caarv1/Caarv1, Caarv1\(^{AHD} \), Caarv1/Caarv1\(^{C3S} \), and Caarv1/Caarv1\(^{C28S} \) cells all

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**Table 3 Log rank \( P \) values**

| Strain #1          | Strain #2          | \( P \)-Value |
|--------------------|--------------------|---------------|
| CaARV1/CaARV1\(^a\) | Caarv1/CaARV1      | 0.96          |
| CaARV1/CaARV1      | Caarv1/Caarv1      | <0.0001       |
| Caarv1/CaARV1\(^b\) | Caarv1/CaARV1\(^{AHD} \) | 0.0003       |
| Caarv1/CaARV1      | Caarv1/CaARV1\(^{C28S} \) | <0.0001       |
| Caarv1/CaARV1      | Caarv1/CaARV1\(^{C3S} \) | 0.0003       |
| Caarv1/CaARV1      | Caarv1/CaARV1\(^{CgARV1} \) | 0.85         |

\(^a\) Log rank \( P \) values are compared between the ARV1/ARV1 strain, and the arv1/ARV1 and arv1/arv1 strains.

\(^b\) Log rank \( P \) values are compared between the arv1/ARV1 strain and the arv1\(^{AHD} \), arv1\(^{C3S} \), arv1\(^{C28S} \), and arv1\(^{CgARV1} \) strains.
showed defects in sterol distribution (Figure 7). The percentage of Caarv1/CaArv1 cells properly localizing their sterol was reduced to ~30% of that seen for CaArv1/CaARV1 cells (Figure 8). CaArv1/CaArv1 cells accumulated large sterol aggregates that were localized centrally (Figure 7, CaArv1/CaArv1; arrows and asterisks). The percentage of Caarv1/C24 cells with hyphal tip-localized sterol was lower than that seen for Caarv1/CaArv1 cells (20%) (Figure 8). Caarv1/C24 cells accumulated aggregates that were situated more at the cell periphery (Figure 7, CaArv1/C24; arrows and asterisks). Caarv1/CaARV1 and Caarv1/C28S cells had the least number of cells localizing their sterol to the hyphal tip (Figure 8, ~10%). They both accumulated sterol aggregates and had a diffuse sterol localization concentrated at the plasma membrane surface (Figure 7). Finally, Caarv1/CgARV1 cells properly distributed and localized their sterol (Figure 7 and Figure 8, Caarv1/CgARV1).

Thus, Caarv1/CaArv1, Caarv1/C24, Caarv1/C28S, and Caarv1/CaARV1 cells lack virulence. These strains also had sterol distribution defects and accumulated what appeared to be sterol aggregates. Thus, we can conclude that there was a direct correlation between cells being avirulent and their lack of ability to properly distribute their sterol during hyphal growth.

**DISCUSSION**

*C. albicans* strains lacking CaArv1 are avirulent, suggesting that Arv1 function has a role in maintaining virulence. CaArv1 contains a CaAHD domain that has within it a zinc-binding motif. Here, we explored whether the CaAHD alone was responsible for the virulence function of CaArv1, and, if so, was the zinc-binding motif necessary for virulence. The CaAHD alone could not replace full-length Arv1 function, suggesting that additional domains outside the AHD play a role in virulence. However, we did find that the CaAHD zinc-binding motif was needed for virulence, as cells containing an intact CaArv1 protein harboring either a Cys5 or Cys28 mutation were avirulent, substantiating the hypothesis that CaAHD function is necessary for virulence but is not sufficient. These data hint at the possibility that the activity of the zinc-binding motif is the critical function associated with CaAHD.

There was a strong association between how virulent a strain was and its ability or inability to distribute sterol. Avirulent Caarv1/C24, Caarv1/C28S, and Caarv1/CgARV1 cells all had defects in sterol distribution and septa formation, and all lacked the ability to localize their sterol to the growing hyphal tip. Interestingly, these mutant strains accumulated several sterol biosynthetic intermediates when compared to wild-type cells. *S. cerevisiae* cells lacking ScArv1 have elevated sterol and phosphatidylserine levels in their plasma membrane (Georgiev et al. 2013; Tinkelenberg et al. 2000), display sterol distribution defects during mating (Villasmil et al. 2011), are unable to mobilize PIP2 (Villasmil et al. 2011), and accumulate unknown sterol intermediates (Swain et al. 2002a). The results in *S. cerevisiae*, along with those presented here, lend strong support to the theory that CaArv1 is highly conserved, and that it regulates sterol distribution during *C. albicans* infection. Just as important, it also strongly suggests that maintaining sterol distribution is critical for *C. albicans* infection.

Organ colonization and invasion are considered major mortality factors, especially in the case of the kidney (Ashman et al. 1996; Fisher et al. 2011; Vecchiarelli et al. 1988). Mice infected with the *C. albicans* arv1C24 strain had normal kidney fungal loads, but survived for the entire length of the study. There are several avirulent *C. albicans* mutants that cause elevated kidney CFUs (Douglas et al. 2009; Epp et al. 2010), so there is precedence for this observation. On the other hand, mice injected with either Caarv1/C24 or Caarv1/C28S cells had reduced fungal loads, and this correlated well with avirulence, suggesting that the zinc-binding motif has a role in organ colonization and invasion. Unexpectedly, we found that mice injected with Caarv1/CgARV1 had reduced organ fungal loads, suggesting an increase in fungal clearance. The reason for this phenotype is unclear to us. One possibility is that the Caarv1/CgARV1 strain acts as a superantigen, causing a rapid response that causes early organ failure. Animals infected with Caarv1/CgARV1 do die much sooner than those infected with other virulent strains. Thus, we may have missed the most appropriate time to demonstrate colonization and invasion. Superantigen effects have been seen during *S. pneumoniae* infection (Tilahun et al. 2014), initiation of toxic shock...
(Hanna and Tierno 1985; Meedt et al. 2010), and Staphylococcus aureus infection (Langley et al. 2010). It is interesting to point out that Caarv1/CaARV1 cells secrete higher levels of aspartyl proteases (P. McCourt, unpublished data). Whether this increased secretion contributes to increased pathogenicity is presently being explored.

Caarv1/CaARV1 and Caarv1/CaARV1AHD cells were delayed in forming hyphae, accumulated large sterol aggregates, and had a reduced number of cells localizing their sterol to the hyphal tip. On the other hand, Caarv1/C3S and Caarv1/C28S cells had a diffuse peripheral sterol-staining pattern, but were also delayed in hyphal formation. S. cerevisiae mating haploids must localize their sterol to the polarized mating projection tip in order to mate (Bagnat and Simons 2002; Jin et al. 2008; Proszynski et al. 2006; Simons and Toomre 2000; Villasmil et al. 2011). Scarv1 cells are sterile, and this correlates with sterol distribution defects and a reduction in mating projection formation (Villasmil et al. 2011). C. albicans cells localize their sterol to cell septa and hyphal tips upon initiating invasive growth (Gallo-Ebert et al. 2012; Martin and Konopka 2004), and this is required for hyphal formation (Chen and Thorner 2007; Sudbery 2011). Thus, both ScArv1 and CaArv1 seem to distribute sterol to sites of membrane clustering and polarization.

Data suggest that Arv1 has the ability to distribute lipids other than sterol, including the glycerophospholipid, phosphatidylserine. Scarv1 mutants are hypersensitive to the phosphatidylserine-binding agent, papuamide B, suggesting a mislocalization of this lipid to the outer plasma membrane. Studies have shown that phosphatidylserine flipping is required for mating projection formation in S. cerevisiae, indicating that phosphatidylserine must be properly localized for maintaining polarized growth (Sartorel et al. 2015). Interestingly, Scarv1 cells have defects in localizing factors required for phosphatidylserine distribution and polarized growth. Scs2 is required for phosphatidylserine transport, and its loss causes phosphatidylserine transport defects, abnormal bud morphology, and sporulation defects (Riekhof et al. 2014). Scarv1 cells cannot properly localize the C-terminal portion of Scs2 to the endoplasmic reticulum. The C. albicans ORF 19.1212 is orthologous to Scs2 (http://www.candidagenome.org/cgi-bin/locus.pl?locus=C6_04100W_B). The orf 19.1212 protein product has a FFAT domain (Hanada et al. 2009), and is proposed

![Figure 5](image5.png)

**Figure 5** Hyphal formation is delayed in strains carrying Caarv1AHD, Caarv1C3S, and Caarv1C28S alleles. Various Caarv1 strains were grown to exponential phase in YEPD at 30°C. Invasive growth was initiated by shifting cultures to 37°C for 3 hr in 10% FBS. Hyphal formation was determined at 3 hr using light microscopy. (A) DAPI stained CaARV1/CaARV1 cells. (B) Percentage of cells forming hyphae. Percentages are the average of five independent experiments. **P < 0.001; ***P < 0.0001.

![Figure 6](image6.png)

**Figure 6** Bud site selection and septa formation are defective in strains carrying Caarv1AHD, Caarv1C3S, and Caarv1C28S alleles. Various Caarv1 strains were grown to exponential phase in YEPD at 30°C. Invasive growth was initiated by shifting cultures to 37°C for 3 hr in 10% FBS. (A, B), Bud site selection was determined at 3 hr by fixing cells in paraformaldehyde and staining with calcofluor white. (A) CaARV1/CaARV1 cells (arrows, septa; asterisk, chitin stained bud site). (B) Caarv1/-/Caarv1 cells (arrows, chitin stained bud site; hash signs, constrictions along the hyphae; asterisk, cells with two chitin stained bud sites). (C) White bars, number of cells with ≥2 chitin bud sites; black bars, number of cells with < 2 chitin bud sites.
to be a lipid transporter. Whether Scs2 is involved in mating, and if 19.1212 is involved in virulence and/or regulates lipid distribution during invasion, remains to be studied. Interestingly, the phosphatidylserine synthase Cho1 and the phosphatidylserine decarboxylase Psd1 have been shown to be required for filamentous growth in *S. cerevisiae* and virulence in *C. albicans* (Chen et al. 2010). Thus, there exists a link between Arv1 function, maintaining proper phosphatidylserine homeostasis, and fungal infection.

Overall, our data strongly suggest that multiple domains of *C. albicans* Arv1 are required for function and virulence. They also indicate that the CaAHD is necessary for virulence, but it alone cannot substitute for full-length CaArv1. Moreover, we have validated the importance of the zinc-binding domain in conferring virulence. AHD homology searches indicate that the AHD and zinc-binding domain are conserved among a large population of pathogenic yeasts. Thus, targeting Arv1 for drug discovery may represent a novel approach for treating systemic candidiasis.

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**Figure 7** Sterol distribution during hyphal development is defective in strains carrying Caarv1AHD, Caarv1C3S, and Caarv1C28S alleles. Various Caarv1 strains were grown to exponential phase in YEPD at 30°. Invasive growth was initiated by shifting cultures to 37° for 3 hr in 10% FBS. Sterol localization was determined at 3 hr by fixing cells in paraformaldehyde and staining with filipin. Sterol localization was visualized by fluorescence microscopy using a Leica DRME microscope. Arrows indicate sterol localization during hyphal growth; asterisks indicate defective sterol localization.

**Figure 8** Sterol localization to the hyphal tip is defective in Caarv1AHD, Caarv1C3S, and Caarv1C28S allele expressing strains. Caarv1 strains were grown to exponential phase in YEPD at 30°. Invasive growth was initiated by shifting cultures to 37° for 3 hr in 10% FBS. Sterol localization was determined at 3 hr by fixing cells in paraformaldehyde and using filipin staining and fluorescence microscopy. Sterol localization was visualized using a Leica DRME microscope. The data are the average of five independent experiments. **P** < 0.001; ***P** < 0.0001.
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