

***Candida albicans* Dap1p Promotes Ergosterol Synthesis via the P450 Protein Erg11p/Cyp51p, Regulating Susceptibility to Azole Antifungal Drugs, Morphogenesis and Damage Resistance**

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Abstract: Background: *Candida albicans* is an opportunistic pathogen in humans and is a significant cause of disease progression and death in immune-compromised patients. One of the most effective drug classes for treating *C. albicans* infections is the azole drugs which inhibit the P450 protein Erg11p/Cyp51p/lanosterol demethylase. Dap1p is part of a widely conserved group of proteins that includes the yeast Dap1p proteins in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the PGRMC1/Hpr6.6/25-Dx proteins in mammals and unnamed homologous in multiple pathogenic fungi. In non-pathogenic yeast and in humans, Dap1p or its homologue activates Erg11p and promotes sterol synthesis. **Results:** The DAP1p family had not been studied in pathogenic yeast and we studied that deletion of both copies of the DAP1p gene in *C. albicans* causes a partial arrest in ergosterol synthesis at the step catalyzed by Erg11p. As a result, loss of DAP1p causes elevated azole drug susceptibility and DAP1 is induced by multiple azole drugs. Furthermore, DAP1p directs filamentous growth in suspension culture and is required for resistance to the DNA damaging agent Methyl Methane sulfonate (MMS). **Conclusions:** Present results support a model in which DAP1p activates an essential reaction in pathogenic fungi, suggesting that DAP1p may be a target for antifungal therapeutics to be used in conjunction with existing therapies.

Key words: *Candida*, DNA damage, sterol, drug resistance, pseudohyphae

INTRODUCTION

Candida infections are a major cause of death in immuno-compromised patients, such as HIV and transplant patients and patients receiving cancer chemotherapy (White *et al.*, 1998). *Candida albicans* infections are typically treated with azole drugs that inhibit the enzyme Erg11p/Cyp51p/lanosterol demethylase (Lepesheva and Waterman, 2007; Waterman and Lepesheva, 2005) or with drugs that the ergosterol biosynthetic pathway. Erg11p is a cytochrome P450 protein (Nebert and Russell, 2002), a class of proteins that was named for its unusual absorption spectrum that arises from bound heme (Werck-Reichhart and Feyereisen, 2000). Erg11p requires a reductase partner and Erg11p activity is elevated by cytochrome b₅, Cyb5p (Lamb *et al.*, 1999; Rogers *et al.*, 2004; Schenkman and Jansson, 2003).

Disrupted ergosterol synthesis triggers alterations in the expression patterns of numerous genes (Agarwal *et al.*, 2003; Bammert and Fostel, 2000;

Hughes *et al.*, 2000), including 11 ERG (ergosterol synthetic) genes, CYB5/cytochrome b₅ (Agarwal *et al.*, 2003), heme synthetic genes and DAP1 (damage resistance protein 1). Dap1p is composed largely of a region of limited homology with cytochrome b₅, called a heme-1 domain (Hand *et al.*, 2003; Mifsud and Bateman, 2002). Cells deleted for DAP1 are defective in ergosterol biosynthesis at the step catalyzed by Erg11p (Hand *et al.*, 2003) and as a result, *dap1Δ* cells are sensitive to azole antifungal drugs (Hand *et al.*, 2003; Mallory *et al.*, 2005a), which inhibit Erg11p. While Dap1p was originally characterized in *S. cerevisiae*, Dap1p has a similar role in the phylogenetically distant yeast *Schizosaccharomyces pombe* (Hughes *et al.*, 2007). Dap1p has been included as part of the global antifungal drug resistance network (Parsons *et al.*, 2004) and likened to a “helping hand” for P450 proteins (Debose-Boyd, 2007).

ERG11 over-expression suppresses itraconazole sensitivity in *dap1Δ* cells and restores normal ergosterol synthesis to *dap1Δ* cells (Mallory *et al.*, 2005a) and in some strain backgrounds, Dap1p regulates the stability of

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Erg11p (Mallory *et al.*, 2005a). Dap1p binds to heme through a penta-coordinate mechanism that utilizes a carboxy-terminal tyrosine (Ghosh *et al.*, 2005) and heme binding is required for sterol synthesis and for stabilizing Erg11p (Mallory *et al.*, 2005a). In addition to regulating sterol synthesis, Dap1p directs resistance to the methylating agent, methyl Methane sulfonate (Hand *et al.*, 2003). MMS sensitivity is also due to defective Erg11p function (Mallory *et al.*, 2005a), suggesting that sterol synthesis is needed for the repair of chemically induced damage, a conclusion that is supported by genome-wide studies (Bennett *et al.*, 2001). Dap1p is also required for the uptake and storage of iron and like the other Dap1p phenotypes, Dap1p-mediated iron metabolism is mediated by Erg11p (Craven *et al.*, 2007).

In *S. cerevisiae*, Dap1p lacks a putative membrane-spanning sequence and localizes to punctuate cytoplasmic sites that co-fractionate with endosomes (Craven *et al.*, 2007). The localization of *S. cerevisiae* Dap1p overlaps partially with that of Erg11p (Craven *et al.*, 2007) but no direct interaction for the two proteins has been reported. In contrast, *S. pombe* Dap1p has a putative amino-terminal membrane spanning sequence and co-precipitates with Erg11p (Hughes *et al.*, 2007), as does the mammalian Dap1p homologue, PGRMC1/Hpr6 (Hughes *et al.*, 2007). PGRMC1 and the related rodent homologous localize to the endoplasmic reticulum (Crudden *et al.*, 2005; Nolte *et al.*, 2000), with a smaller fraction localizing to the cell membrane (Krebs *et al.*, 2000).

Because *S. cerevisiae* and *S. pombe* Dap1p regulate azole drug resistance and iron metabolism, pathways that are important for the biology of pathogenic yeast, we have tested the extent to which *C. albicans* Dap1p performs analogous functions. We have found that, like its relatives in non-pathogenic yeast, *C. albicans* Dap1p regulates ergosterol synthesis at the step catalyzed by Erg11p and promotes azole drug resistance. Furthermore, *C. albicans* Dap1p is required for filamentous growth and for resistance to the DNA damaging agent, methyl methane sulfonate (MMS). Thus, *C. albicans* Dap1p activates an essential pathway in pathogenic fungi that regulates drug susceptibility and damage resistance.

MATERIALS AND METHODS

Culture conditions and chemicals: Unless stated otherwise, cells were maintained by culturing in Yeast Peptone-Dextrose (YPD) medium at 30°C. Fluconazole (LKT laboratories), ketoconazole (Sigma), itraconazole (Sigma), PD98059 (Calbiochem), methyl, methane sulfonate (Sigma) and ferrozine (Acros) were added at the concentrations indicated in the text.

Strain construction: All strain manipulations were performed in the wild-type strain BWP17 (Wilson *et al.*, 1999). The first copy of DAP1 was deleted by targeted integration of the plasmid pJM72 digested with Hind III and Apa I. In all cases, cells were transformed using the standard lithium acetate transformation procedure, except that cells were incubated for 1 h at 30°C in YPD media before plating. We used this approach because we identified unusual recombination events at the 3' end of the DAP1 gene when smaller regions of homology were used. We did not detect these events during the deletion of the second copy of DAP1 with the HIS3 gene. The disruption of the DAP1 gene by URA3 was verified initially by PCR using the primers CaDAP1-1081F and CaURA3-154R for the 5' end and CaURA3+750F and CaDAP1+1600R for the 3' end. Oligonucleotide sequences are shown in (Fig. s1). The DAP1/dap1Δ::URA3 strain was named JMCa3.

The second copy of DAP1 was deleted by transformation of JMCa3 with a PCR product generated by amplification of the HIS1 gene from the plasmid pFA-HIS1 (Gola *et al.*, 2003) with the primers CaDAP1-54-KOF and CaDAP1+573-KOR. The deletion of DAP1 was verified initially by PCR using the primers CaDAP1-1081F, CaHIS1-280R, CaHIS1+983F and CaDAP1+1600R. The dap1Δ::URA3 dap1Δ::HIS1 strain was named JMCa5. All of the primers are outside the region of homology targeted by the integration. To re-introduce the DAP1 gene to dap1Δ/dap1Δ cells, the plasmid pRC65 was digested with BbsI and used to transform JMCa5 cells. Arg⁺ colonies were tested for re-integration using the primers CaDAP1-1081F and CaDAP1+488R-Xho.

Plasmids: The CaDAP1 deletion plasmid pJM72 was prepared as follows. (1) The URA3 gene was amplified from pFA-URA3 (Gola *et al.*, 2003) with the primers CaURA3-220F and CaURA3+940R and sub-cloned into the plasmid pCR2.1 (Invitrogen), forming the plasmid pJM65. (2) The 1 kb of genomic DNA flanking the 5' end of DAP1 was amplified from BWP17 genomic DNA using the primers CaDAP1-1000F and CaDAP1-1R and sub-cloned into the Hind III and Sac I sites of pJM65, forming the plasmid pJM70. (3) The 1 kb of genomic DNA flanking the 3' end of the DAP1 gene was sub-cloned into pCR2.1 using the primers CaDAP1+469F and CaDAP1+1468R, forming the plasmid pJM67. (4) The Not I-Apa I fragment containing the DAP1 flanking sequence from pJM67 was sub-cloned into the same sites of plasmid pJM70, forming the plasmid pJM72.

The CaDAP1 knock-in plasmid pRC65 was constructed as follows. The CaDAP1 open reading frame, along with 1000 bp of upstream sequence, was PCR amplified using the primers CaDAP1-1000F-Hind and

<i>CaDAP1</i> -1081F	GGAGTCTTTGATAAATACTTGCAGCAG
<i>CaDAP1</i> -1000F	CCCAAGCTTGCTTTTATCCCCCCACAGGC
<i>CaDAP1</i> -54-KOF	TAACATTAATACTACTATTACTATTGTTTTATATCAACT AAAAGAACTACAGTCATGCTTACTACAATTCTTATTAT ACTCATTATTTTGATTTTGCCGAAAGCTTCGTACGCTGCA GGT
<i>CaDAP1</i> -12F-Bam	GTCGTCAAGGGGGGATCCGAAACTACAGTCATGCTTAC TACAATTCTTATTATA
<i>CaDAP1</i> -1R	CCCGAGCTCGACTGTAGTTTCTTTTAGTTG
<i>CaDAP1</i> +2F	CTTACTACAATTCTTATTAT
<i>CaDAP1</i> +250R	GTCCACCTGGTCCATAAAATG
<i>CaDAP1</i> +469F	AATGCGGCCGCGTATTGACTAGTATAGAACGA
<i>CaDAP1</i> +488R	CCGCTCGAGCGTTCTATACTAGTCAATAC
<i>CaDAP1</i> +573-KOR	TTGTTAGGCAATTGTAAAGACCATCTATTATTAGATAC ATAGTAGTGTTAATAAAAGCTGACTGAAATAAACAA GAAATACTATCGTTCTATACTAGTCAATACTCTGATAT CATCGATGAATTCGAG
<i>CaDAP1</i> +579R- <i>Pst</i>	GGGTATACCAGACTGCAGCATTCTTTGTTAGGCAATTG TAAAGACCATCTATTA
<i>CaDAP1</i> +1468R	AATCCCGGGGCTGTC AACGTCCAATTTCA
<i>CaDAP1</i> +1600R	CTCCATGTTTCGAAAACCTTTGATCTTGGTG
<i>CaHIS1</i> -280R	CTTCTTCCTCTGATGTCACT
<i>CaHIS1</i> +983F	ATCTACCACCTTGATGTACAC
<i>CaTUB1</i> +421F	TGATTTGGAACCAAATGTCAT
<i>CaTUB1</i> +600R	GATCACTCATTCTTCTGACTC
<i>CaURA3</i> -220F	CCCGGATCCTCCAAAAAATTGATTCGTA
<i>CaURA3</i> -154R	CTTTCATTATGTATCTGTATAATATG
<i>CaURA3</i> +750F	TCCATAGAGATGCTGGTTGGAATGC
<i>CaURA3</i> +940R	CCCAAGCTTTAGAAGGACCACCTTTGATTC

Fig. s1: Oligonucleotide sequences used in this study

CaDAP1+488R-Xho cloned into plasmid pCR2.1 (Invitrogen), resulting in the plasmid pRC63. A NotI fragment containing the ARG4 gene from the plasmid pFA-ARG4 was then cloned into the NotI site of pRC63, forming the plasmid pRC65.

Spotting assays and plate preparation: For all spotting assays, cells were serially diluted 1:10 in water and spotted on plates containing 10 μ M fluconazole, 0.02% methyl Methane sulfonate, or 100 μ M bathophenanthroline. Colonies were photographed after 48 h incubation at 30°C. The growth of the *dap1 Δ /dap1 Δ* strain JMCa5 was compared with that of CNC44, an Arg⁻ Ura⁺ His⁺ SC5314 derivative (Negredo *et al.*, 1997). For halo formation assays, cells were grown to log phase (approximately A600 = 1), whereupon the A600 was measured. Cells were diluted to 0.3 A600 units in 3 mL of water containing 0.7% of melted Bacto-agar that was maintained at 48°C. The cell suspension was immediately spread on YPD plates and allowed to harden. Paper disks were placed on the agar and 10 μ L of various drugs were spotted on the disks. The plates were then incubated for 24-48 h at 30°C and photographed.

Sterol analysis: Sterol profiles were analyzed by the KOH/n-heptane extraction procedure of Molzahn and

Woods (Molzahn and Woods, 1972) as previously described (Hand *et al.*, 2003). Cells were grown in liquid medium and harvested for sterol analysis at an A600 of 0.5-1. Cells were centrifuged, washed once with dH₂O and resuspended in 4.5 M KOH/60% ethanol. Cells were then heated at 88-90°C for 1 h in a round-bottom flask. Ethanol (95%) was then added and the cells were heated for an additional hour and cooled to room temperature. The mixture was then extracted with n-heptane and dH₂O and the n-heptane layer was analyzed by gas chromatography at the University of Kentucky GC-MS facility.

Morphological analysis: Approximately 1000 cells mL⁻¹ were plated on YPD containing 10% fetal bovine serum. The plates were incubated at 37°C for 7 days. For filamentous growth, log phase cells were suspended in molten YPS agar (1% yeast extract, 2% bacto-peptone, 2% sucrose and 2% agar) at a concentration of 100 cells/mL and plated. After 1-3 days at 37°C, the colonies were photographed. In other experiments, cells were suspended in spider media [1% nutrient broth, 0.2% K₂HPO₄, 1.35% agar and 1% mannitol (Lee *et al.*, 1975)].

Expression analysis: RNA was isolated from log phase yeast cells using the RNeasy kit from Qiagen using the manufacturer's instructions, except that cells were

spheroplasted in 1 M sorbitol, 100 mM Tris, pH 7.8 and 100 mM EDTA containing 150 µg zymolase. Three micrograms of RNA was reverse transcribed as described previously (Mallory *et al.*, 2005a). The ratios of DAPI:TUB1 (an internal control for cDNA loading) were determined using the primers CaDAP1+2F, CaDAP1+250R, CaTUB1+421F and CaTUB1+600R in the same reaction. PCR reactions were separated on a 1.5% agarose 1000 gel (InVitrogen).

RESULTS

The DAP1 gene conserved among fungi: Dap1p has homologous in virtually all fungi, including pathogenic fungi such as the *Candida* species, *Aspergillus* and *Cryptococcus* (Fig. 1). All of the key sequences in ScDap1p are conserved in this protein

family, including the strictly conserved FYGPxGPYxNFAGxDASRGLA motif at the heart of the heme-binding domain (Fig. 1, center). The *S. cerevisiae* Dap1p Asp91 and Tyr 138 are required for heme binding and both residues are conserved among all of the fungal Dap1p homologous (Ghosh *et al.*, 2005; Mallory *et al.*, 2005a). However, some Dap1p homologous lack a hydrophobic membrane-spanning sequence at their amino-termini, while others, including CaDap1p, contain this sequence. In this way, CaDap1p resembles its mammalian homologous (Falkenstein *et al.*, 1996; Gerdes *et al.*, 1998; Krebs *et al.*, 2000; Meyer *et al.*, 1998; Nolte *et al.*, 2000; Selmin *et al.*, 1996).

The entire open reading frames of each copy of DAP1 were replaced with the URA3 and HIS1 genes, resulting in the *dap1Δ::URA3/ dap1Δ::HIS1* strain JMCa5

Group A-TM domain sub-family

<i>C.alb</i> :	MLTT	ILILILYFARNIY	NEFINNPSSPLDVSTEKESTIVEGK	ETISPKYNSKDSPKI
<i>A.nid</i> :	MS(14)	TPENLILFSAFALV	YTQFRPKPPAVLPKAFAPV	VFRTETKTLFFNGENKPV
<i>A.fum</i> :	MSIA	TPENLILLSLFAILV	YMQLRPKAPVALPQAFPPV	VFRTETTTLEYNGEGKPV
<i>C.neo</i> :	MSLSNP	LNLLLVVPLLEFLAYR	ILVPPPPHTPP (19)	EAQLAQYDTNGDRILAIMRVAPDGKI

Group B-non-TM sub-family

<i>S.cer</i> :	MSFI	KNLLFGGVKTS	EDPTGLTG-NGASNTNDSNKGSEFV	AGNCFERTLSKFNCHDDEKI
<i>C.gla</i> :	MSFL	KNLLFGGVKTS	EDPTGLTD-SGSGNAKASQDVNEPI	VEGRTEERTLSKFNCHDDEKI

Group A

<i>C.alb</i> :	FIDVKNR	YFDVYQEGAYGPGCPYENFACRDASRGLAKNSHEDDY	ETDIN-EIITTTTDT
<i>A.nid</i> :	YLAVRGR	YFDVYPCENYGGPGPYLNFACRDASRGLAFQSHQKEM	LEEDLSGLEDIKDIN
<i>A.fum</i> :	YLAVRGR	YFDVYPCENYGGPGPYLNFACRDASRGLAFQSHQKEM	LEKDLKGLLEDIKDIN
<i>C.neo</i> :	DPNGERT	YFDVYASHTYGRDGVYGNFACRDASRGMKQSEPEV	LETSID-EIITDDESDIT

Group B

<i>S.cer</i> :	FIDLRGK	YFDCTRGHCFYGFSGPYTFNFACHDASRGLAINSEDLDY	TKDWD-CIIDPDDDT
<i>C.gla</i> :	FIDLRGK	YFDCTAGCFYGFSGPYTFNFACHDASRGLAINSEEMEV	VPDWD-KIMDDIKDIT

Group A

<i>C.alb</i> :	AVERESIDGMEEHENRKYVGT
<i>A.nid</i> :	ADQLENQSWERRLEKYLIVCKIYAE
<i>A.fum</i> :	EEQLENQSWERRLEKYLIVCKIYVAGDPEAPKS
<i>C.neo</i> :	PSEIENMRGWHQHRECKYIVCEIIV

Group B

<i>S.cer</i> :	KEQIDADDEWQEHENKYPCTITLIPFPGYV
<i>C.gla</i> :	PQQLDSINEMEQHANKYFVETLEAFPGYV

Fig. 1: Dap1p is part of a highly conserved family of fungal proteins. Proteins are aligned in two groups based on the presence of an amino-terminal putative trans-membrane sequence. *Candida albicans* Dap1p is aligned with homologous from *Aspergillus nidulans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*, while the *Saccharomyces cerevisiae* homologue is aligned with *Candida glabrata* Dap1p. Residues that are shared by both groups are shown in dark gray, while residues shared within either group are highlighted in light gray. The amino-terminal putative trans-membrane sequences and the central heme-1 domain homologies are boxed and asterisks mark residues that are required for heme binding

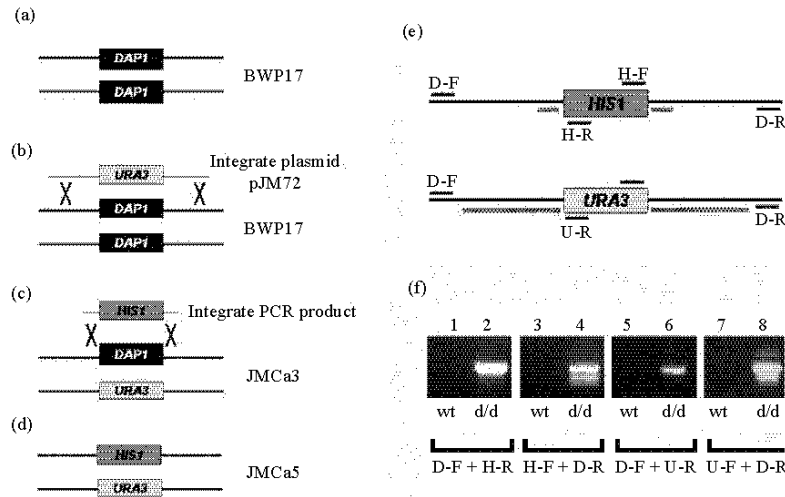


Fig. s2: Deletion of both copies of the *C. alicans* DAP1 gene. One copy of DAP1 (a) was initially deleted by integrating the plasmid pJM72, containing 1 kb of DNA flanking the DAP1 open reading frame adjacent to URA3 (b) resulting in strain JMca3 (c) the second copy of DAP1 was then deleted using a PCR product consisting of the HIS1 gene adjacent to 100 bp of DNA adjacent to the 5' and 3' ends of the DAP1 open reading frame. The *dap1Δ/dap1Δ* strain is called JMca5 (d) the deletion of DAP1 was tested by PCR using primers within the inserted auxotrophic marker (H-F and H-R for HIS1 and U-F and U-R for URA3) and outside the region of homology (grey stippled line) used to target the integration event (primers D-F and D-R). The panels in (f) show PCR products using the primers shown in part (e) with wild-type (wt) DNA from the strain BWP17 and *dap1Δ/dap1Δ* DNA from the strain JMca5 as template. The JMca5 strain was subsequently used for analyzing azole drug sensitivity, ergosterol synthesis and invasive phenotypes

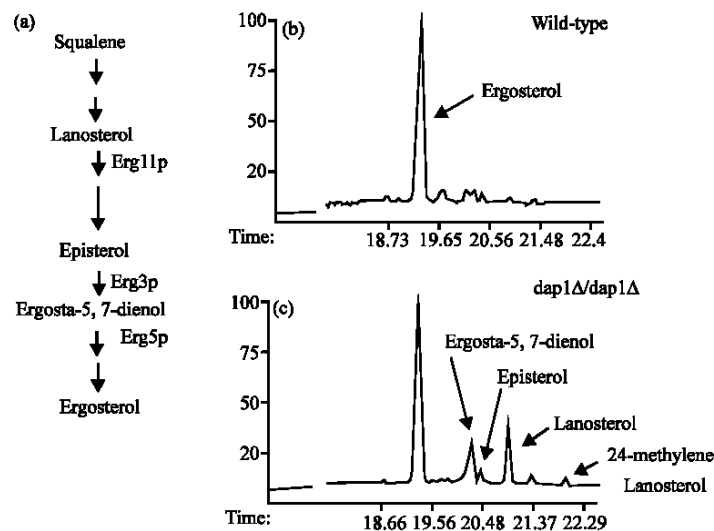


Fig. 2 (a-c): Mutants lacking Dap1p have altered levels of sterol metabolites. (a) Depiction of the sterol biosynthetic pathway showing the relevant intermediates. (b) The sterol profiles of wild-type CNC44 or *dap1Δ/dap1Δ* JMca5 and (c) cells were analyzed by gas chromatography, showing increased peaks for lanosterol, 24-methylene lanosterol, ergosta-5,7- dienol and episterol in the *dap1Δ/dap1Δ* strain. For b and c, the X axis represents retention time and the Y-axis represents relative abundance

(Fig. s2). The *dap1Δ/dap1Δ* strain grew at a wild-type rate under normal growth conditions and appeared morphologically normal microscopically, similar to analogous strains in other yeast species. JMCa5 did not exhibit temperature sensitivity or defective growth on synthetic media.

The *C. albicans* *dap1Δ/dap1Δ* mutant has increased azole susceptibility: The *dap1Δ/dap1Δ* strain JMCa5 were measured using gas chromatography. Ergosterol is synthesized via a multi-step pathway that includes the first sterol intermediate, lanosterol (Fig. 2a). Compared to the wild-type strain (Fig. 2b), JMCa5 exhibited a marked increase in lanosterol and a smaller increase in 24-methylene lanosterol (Fig. 2c), suggesting a partial arrest at the step of ergosterol synthesis catalyzed by Erg11p. In addition, *dap1Δ/dap1Δ* cells accumulated the Erg5p and Erg3p substrates ergosta-5,7-dienol and episterol, respectively (Fig. 2c).

The *C. albicans* *dap1Δ/dap1Δ* strain JMCa5 grew poorly on plates containing 2-20 μM fluconazole compared to the strain CNC44 (Fig. 3a) which was used because its auxotrophy is identical to JMCa5 (Negredo *et al.*, 1997). In addition, *dap1Δ/dap1Δ* cells were hyper-sensitive to the Erg11p inhibitors itraconazole and ketoconazole in spotting assays (Fig. 3a, respectively) and in halo-forming assays for fluconazole (Fig. s3). In a liquid growth assay, the *dap1Δ/dap1Δ* strain had a 9-fold lower MIC₈₀ for fluconazole than the wild-type strain (2.0 versus 18.2 μM) which was highly significant ($P = 6 \times 10^{-5}$, two-tailed t-test). Microscopic analysis revealed enlarged, elongated buds after fluconazole treatment in the *dap1Δ/dap1Δ* strain that were not detected in wild-type cells (Fig. s3). The fluconazole sensitivity of the *dap1Δ/dap1Δ* strain was complemented by the insertion of the wild-type DAP1 gene (Fig. 3b). Thus, like its *S. cerevisiae* and *S. pombe* analogues, *C. albicans* strains lacking ergosterol synthesis (Hand *et al.*, 2003; Hughes *et al.*, 2007).

We identified the role for *S. cerevisiae* DAP1 in ergosterol synthesis, in part, through its transcriptional induction by azole antifungal drugs. We used PCR to test the expression of *C. albicans* DAP1 after treatment with various azole drugs for 3 h and detected a 1.7-2.0-fold induction in the DAP1 transcript after treatment with fluconazole, itraconazole and ketoconazole (Fig. 4a). No DAP1 band was detectable in cDNA from the *dap1Δ/dap1Δ* strain JMCa5 (Fig. 4a) and primers for the *C. albicans* tubulin gene TUB1 were included in each reaction to control for sample loading (Fig. 4a). DAP1 regulation has been reported to be under the control of

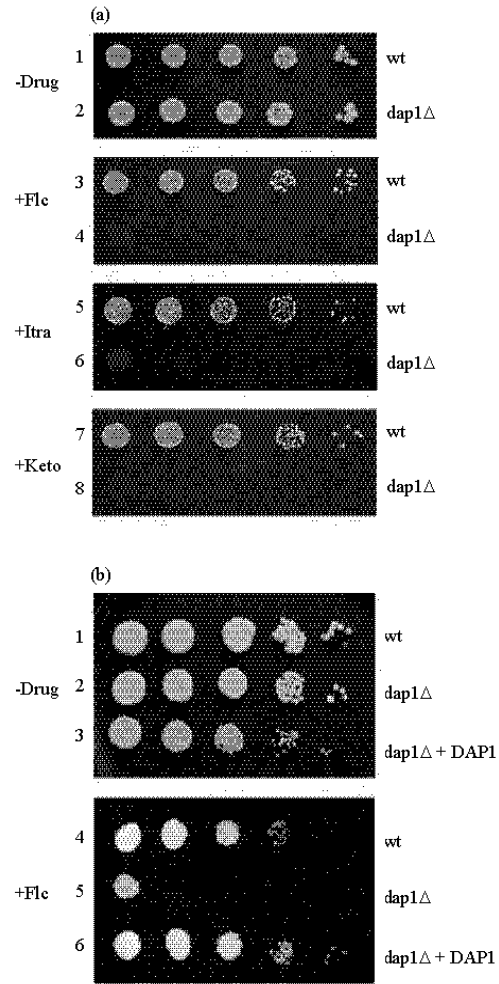


Fig. 3 (a-b): *C. albicans* *dap1Δ/dap1Δ* strains have increased azole drug susceptibility. (a) The wild-type CNC44 strain or the *dap1Δ/dap1Δ* strain JMCa5 were plated on YPD plates without (rows 1-2) or with 10 μM fluconazole (rows 3-4), 10 μM itraconazole (rows 5-6) or 10 μM ketoconazole (rows 7-8) and (b) Fluconazole sensitivity in the *dap1Δ/dap1Δ* strain JMCa5 (row 5) was complemented by an inserted copy of DAP1 (row 6)

the MAP kinase Hog1p and the addition of a MAP kinase inhibitor, PD98059, inhibited the induction of DAP1 from 1.9-fold with fluconazole to baseline levels (Fig. 4b). These results suggest that MAP kinases may contribute to the regulation of DAP1 by azole drugs.

Dap1p regulates filamentous growth: Because Dap1p regulates sterol synthesis in *C. albicans*, we tested the ability of *dap1Δ/dap1Δ* mutants to undergo filamentous

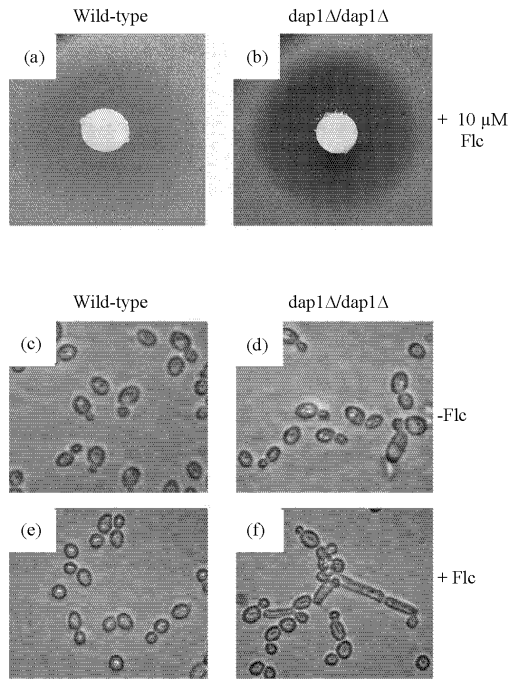


Fig. 3 (a-f): Dap1p-associated azole susceptibility phenotypes. Wild-type (a) and *dap1A* (b) were tested for azole susceptibility by halo formation assay in which paper disks were saturated with 5 l of 10 M fluconazole. The *dap1Δ* strain had decreased residual growth after treatment. The morphology of the wild-type CNC44 strain changed little after 3 h of fluconazole treatment (c and d), while the *dap1A* strain JMCa5 developed an increased number of elongated cells (e and f)

growth. The wild-type CNC44 and the *dap1Δ/dap1Δ* JMCa5 strains were plated on media containing 10% serum and incubated at 30°C for 7 days. The wild-type strain formed colonies with a ruffled appearance (Fig. 5a), while the *dap1Δ/dap1Δ* strain formed a larger proportion of smooth colonies (Fig. 5b). When grown in suspension in YPS medium, wild-type cells formed foci with prominent filaments (Fig. 5c), while foci of the *dap1Δ/dap1Δ* strain were generally smooth, with a few single filaments detectable (Fig. 5d). Wild-type and *dap1Δ/dap1Δ* strains formed similar structures when suspended in Spider media (Fig. 5e, f). However, when the same strains were grown in liquid spider media or RPMI-1640, there was a modest difference in morphologies (data not shown), suggesting that cells lacking DAP1 switch to a filamentous morphology under specific growth conditions.

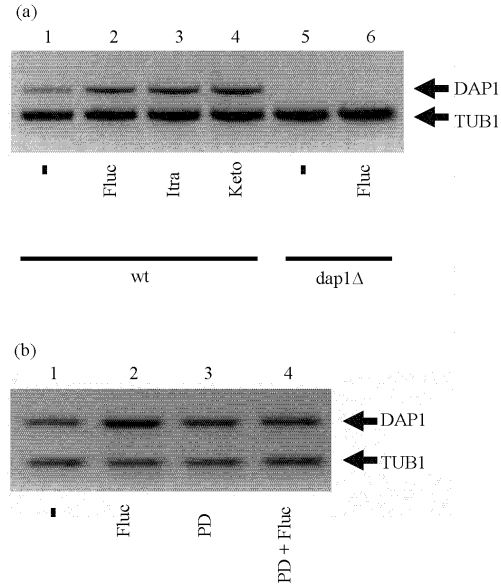


Fig. 4(a-b): DAP1 transcription is induced by azole drugs. (a) DAP1 expression in the wild-type SC5314 strain after 3 h of 10 μM fluconazole (lane 2), 10 μM itraconazole (lane 3) or 10 μM ketoconazole (lane 4) treatment. Expression was measured by reverse-transcriptase PCR using primers to the TUB1 gene in the same PCR reaction as controls for cDNA loading (lower bands). For lanes 1-4, the DAP1:TUB1 ratios were 0.21, 0.36, 0.37 and 0.41, respectively. The *dap1Δ/dap1Δ* strain JMCa5 was used to test the identity of the DAP1 band (lanes 5 and 6), before or after the same treatments as were used in lane 2. (b) DAP1 induction by 10 μM fluconazole after 3 h treatment (lane 2) was reversed by the addition of the MAP kinase inhibitor PD98059 at 10 μM (lane 4), while 10 μM PD98059 had no effect by itself (lane 3). For lanes 1-4, the DAP1:TUB1 ratios were 1.11, 2.13, 1.47 and 1.40, respectively. The analysis was performed using the same conditions described in (a)

MMS sensitivity and low iron growth: Dap1p mediates resistance to the DNA damaging agent MMS in *S. cerevisiae*. The *dap1Δ/dap1Δ* strain JMCa5 was markedly sensitive to MMS compared to the wild-type CNC44 strain (Fig. 6a). On the iron chelating agent ferrozine, wild-type strains grew normally and retained their white color, while *dap1Δ/dap1Δ* strains became dark red, although their growth was not affected (Fig. 6b).

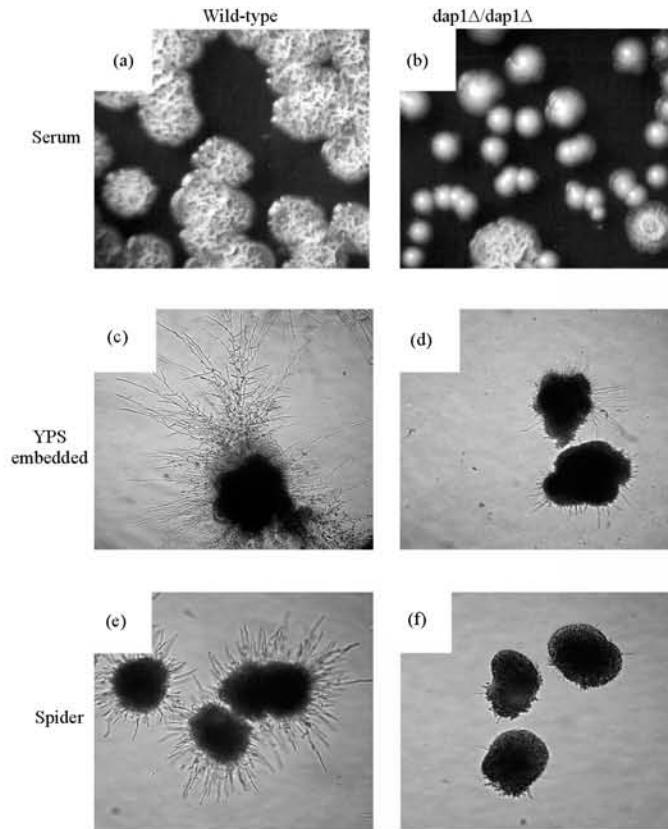


Fig. 5 (a-f): *C. albicans* cells lacking DAP1 have an altered morphology. The wild-type strain CNC44 (a, c and e) or the *dap1Δ/dap1Δ* strain JMCa5 (b, d and f) were plated on media containing 10% serum (a and b) or were resuspended in YPS (yeast-peptone- sucrose, c and d) or spider media (e and f)

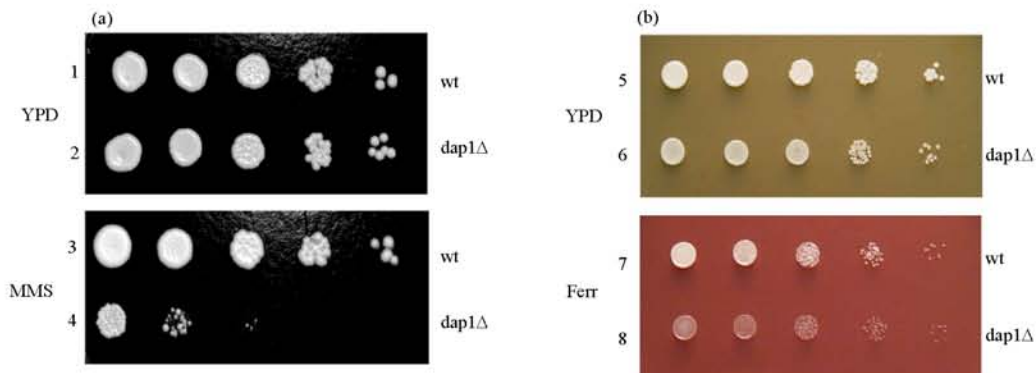


Fig. 6 (a-b): MMS sensitivity in cells lacking Dap1p. (a) The wild-type CNC44 strain or the *dap1Δ/dap1Δ* strain JMCa5 were plated on YPD plates without (rows 1-2) or with 0.015% MMS (rows 3-4) and (b) The same strains were plated on YPD without (rows 5-6) or with 800 μ M ferrozine (rows 7-8)

DISCUSSION

C. albicans Dap1p is the first member of the Dap1p/PGRMC1 family to be characterized in pathogenic fungi. *C. albicans* cells lacking Dap1p accumulate lanosterol, suggesting that Dap1p is required for wild-type Erg11p function and are hyper-sensitive to inhibitors of the ergosterol biosynthetic pathway, like comparable strains in *S. cerevisiae* and *S. pombe* (Hand *et al.*, 2003; Hughes *et al.*, 2007; Mallory *et al.*, 2005a). It is possible that azole susceptibility is due, in part, to the elevated levels of lanosterol in *dap1Δ* strains, rather than decreased ergosterol synthesis. However, the targets of elevated lanosterol in *C. albicans* are largely unknown. In addition to lanosterol, *C. albicans* *dap1Δ* mutants accumulate ergosta-5, 7-dienol, the Erg5p/sterol C-22 desaturase substrate and episterol, the substrate of Erg3p/sterol C-5 desaturase. This effect was detected in *S. cerevisiae* (Hand *et al.*, 2003; Mallory *et al.*, 2005a), suggesting that this is a conserved function of Dap1p. Both the sterol C-5 desaturases and sterol C-22 desaturases are activated by cytochrome *b₅*, with which Dap1p shares homology (Mifsud and Bateman, 2002). Like Erg11p, Erg5p is a P450 protein while Erg3p contains sequences for iron binding and Dap1p has been implicated in the transport or storage of iron (Craven *et al.*, 2007).

In addition to sterol defects and increased azole susceptibility, one of the most pronounced phenotypes associated with Dap1p was diminished filamentous growth. In the early stages of the assay, filaments were notably absent (Fig. 4) and although *dap1Δ* colonies were ultimately able to form mycelae, the filaments were smaller than the wild-type strain and contained clusters of unbranched cells. Ergosterol synthesis is important in hyphal formation (Martin and Konopka, 2004). The association between Dap1p and invasiveness may be important for mammalian cells, because the Dap1p homologue, PGRMC1, is over-expressed in clinical tumor samples (Crudden *et al.*, 2005; Irby *et al.*, 2005) and invasiveness is critical in cancer formation. Furthermore, PGRMC1 is expressed in neuronal cells following damage (Guennoun *et al.*, 2007; Labombarda *et al.*, 2003) and may contribute to the migration and morphology of those cells.

The best characterized function of the Dap1p proteins is heme binding. The Dap1p/PGRMC1 proteins were originally identified as progesterone binding proteins but progesterone binding was not detected for recombinant forms these proteins (Min *et al.*, 2005). In contrast, multiple labs have reported heme binding for Dap1p/PGRMC1 and in a particularly elegant series of studies, *S. cerevisiae* Dap1p was shown to bind to

heme through a 5-coordinate mechanism that utilizes a conserved carboxy-terminal tyrosine residue (Ghosh *et al.*, 2005). Heme binding has also been reported for the *S. pombe* (Hughes *et al.*, 2007), rodent (Min *et al.*, 2005) and human (Crudden *et al.*, 2006; Ghosh *et al.*, 2005) homologous. Nonetheless, there is some evidence that the human Dap1p homologue has a role in progesterone signaling, perhaps through a co-precipitating protein (Peluso *et al.*, 2007). We were unable to detect any difference in proliferation or morphology following treatment of wild-type and *dap1Δ* strains with progesterone.

S. cerevisiae and *S. pombe* Dap1p regulate MMS susceptibility and we have shown that *C. albicans* Dap1p shares this activity. In *S. cerevisiae*, MMS resistance can be restored to *dap1Δ* mutants through over-expression of the heme biosynthetic proteins Hem1p and Hem2p and by adding exogenous heme (Craven *et al.*, 2007; Mallory *et al.*, 2005a), suggesting that MMS may target the heme biosynthetic pathway. Because Erg11p binds heme, one of the ultimate targets of MMS may be Erg11p and we previously showed that Erg11p over-expression suppresses MMS sensitivity in *dap1Δ* mutants (Mallory *et al.*, 2005a). This activity is likely conserved between yeast and humans, because the human Dap1p homologue, Hpr6/PGRMC1, is induced by DNA damaging agents (Mallory *et al.*, 2005b) and promotes survival from DNA damage (Crudden *et al.*, 2006).

The mechanism through which Dap1p or its homologous activate Erg11p is under investigation. The *S. pombe* and human homologous bind directly to P450 proteins (Hughes *et al.*, 2007), although this has not been demonstrated in other organisms and the human homologue activates P450 activity in an over-expression system (Min *et al.*, 2005). Thompson *et al.* recently reported that *S. cerevisiae* Dap1p has a reducing activity similar to that of P450 reductases (Thompson *et al.*, 2007). However, Dap1p has a poor affinity for ferrous heme, which is inconsistent with a direct role in redox cycling (Thompson *et al.*, 2007). These results, together with the findings of the current study, suggest that Dap1p represents a novel regulatory mechanism for P450 proteins such as Erg11p. Thus, Dap1p may represent a novel approach to targeting the most tractable pathway in pathogenic fungi.

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