Regulation of Iron Homeostasis Mediated by the Heme-binding Protein Dap1 (Damage Resistance Protein 1) via the P450 Protein Erg11/Cyp51*

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Fungal infections arise frequently in immunocompromised patients, and sterol synthesis is a primary pathway targeted by antifungal drugs. In particular, the P450 protein Erg11/Cyp51 catalyzes a critical step in ergosterol synthesis, and the azole class of antifungal drugs inhibits Erg11. Dap1 is a heme-binding protein related to cytochrome b₅, which activates Erg11, so that cells lacking Dap1 accumulate the Erg11 substrate and are hypersensitive to Erg11 inhibitors. Heme binding by Dap1 is crucial for its function, and point mutants in its heme-binding domain render Dap1 inactive for sterol biosynthesis and DNA damage resistance. Like Dap1, the human homologue, PGRMC1/Hpr6, also regulates sterol synthesis and DNA damage resistance. In the present study, we demonstrate that the Dap1 heme-1 domain is required for growth under conditions of low iron availability. Loss of Dap1 is suppressed by elevated levels of Erg11 but not by increased heme biosynthesis. Dap1 localizes to punctate cytoplasmic structures that co-fractionate with endosomes, and Dap1 contributes to the integrity of the vacuole. The results suggest that Saccharomyces cerevisiae Dap1 stimulates a P450-catalyzed step in sterol synthesis via a distinct localization from its homologues in Schizosaccharomyces pombe and mammals and that this function regulates iron metabolism.

Fungal infections are important clinically because they contribute to the mortality of patients with human immunodeficiency virus/AIDS, cancer, and other diseases associated with immunosuppression. Mammalian hosts combat fungal infections via the immune system and by sequestering free iron in the bloodstream. Fungal infections can be suppressed with the azole group of antifungal drugs, a commercially important group of drugs that includes fluconazole, itraconazole, and miconazole. These drugs inhibit Erg11/Cyp51/lanosterol demethylase (1, 2), which catalyzes a critical step in the synthesis of ergosterol, a key component of the fungal cell membrane. Erg11 is one of a large class of monoxygenases that are called P450 proteins due to the spectral absorbance of a cysteine-linked heme molecule in their active site (3).

In Saccharomyces cerevisiae, Erg11 is activated by Dap1 (damage resistance protein 1) (4), which is related to cytochrome b₅ (5), a heme-binding protein that activates P450 reactions (3, 6). Cells lacking Dap1 partially arrest sterol synthesis at the stage catalyzed by Erg11 (4), and dop1Δ cells are hypersensitive to the Erg11 inhibitors itraconazole and fluconazole (4). According to microarray databases, DAPI expression is induced by azole antifungal drugs (7), but this has not been independently confirmed. Azole sensitivity in dop1Δ cells is suppressed by overexpressing Erg11 (8), and dop1Δ cells have ∼4-fold lower levels of Erg11 than wild-type cells (8), although this regulation occurs primarily at the post-transcriptional level. Notably, the effect of Dap1 on sterol synthesis is conserved with its human homologue (9), called PGRMC1 (for progesterone receptor membrane component 1) or Hpr6 (for heme-1 domain protein/human progesterone receptor) (10).

Dap1 binds to heme (8, 11), as does its homologues in Schizosaccharomyces pombe, mice, and humans (9, 12, 13). The heme-binding activity of Dap1 is critical for its function, and heme-binding defective mutants are inactive in sterol synthesis or damage resistance (8). Furthermore, the damage sensitivity and sterol synthesis phenotypes of dop1Δ mutants can be suppressed by adding exogenous heme (8), suggesting a role for Dap1 in maintaining heme metabolism. One appealing model for Dap1 family proteins is that they utilize their heme-binding activity to directly activate P450 proteins. However, unlike cytochromes and related proteins, Dap1 homologues lack the histidine residues that coordinate heme binding in cytochrome b₅ (5), suggesting that Dap1 may participate in intracellular heme trafficking. In addition to regulating ergosterol synthesis, Dap1 is required for resistance to the alkylating agent methyl methanesulfonate, and dop1Δ cells have decreased mitochondrial function (4).

Heme (iron protoporphyrin IX) is synthesized in an eight-step pathway that is subject to regulation at various steps (14). Two key steps in heme synthesis are catalyzed by Hem1/5-aminolevulinate synthase, a mitochondrial protein, and Hem2/δ-aminolevulinate dehydratase/porphobilinogen synthase, which localizes to the cytoplasm and nucleus. Heme and ergosterol share the same upstream precursors (15), and the synthesis of heme and ergosterol are closely synchronized. The Hap1 transcription factor (16) is directly regulated by heme through a series of heme-regulated sequence motifs that control multi-

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protein complex formation and DNA binding (17). Heme is also required for the transcription of iron transport and sterol synthetic genes (18). Iron levels, in turn, regulate the transcription, post-transcriptional stability, and post-translational levels of numerous genes and gene products (19–21). One of the iron-regulated transcripts is DAP1, which is post-transcriptionally regulated by proteins that respond to low iron conditions (19).

Because of the close relationship between iron metabolism and the synthesis of heme and ergosterol, we have examined the role of Dap1 in these processes. We have found that Dap1 regulates growth under low iron conditions through a mechanism that requires its heme-1 domain, and Dap1-mediated growth on low iron is mediated by Erg11. We have also shown that Dap1 localizes to endosomes and regulates the structure of the vacuole, which is characteristic of other sterol biosynthetic proteins. The results represent a novel function for the Dap1 family proteins, which include homologues in mammals that are important for sterol synthesis and in cancer.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—All strains were isogenic with W303 (leu2-3,112 his3-11,15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535) (22). The rad5-535 allele was replaced with the wild-type RAD5 gene by crossing and tested by PCR as described (23). Cells were maintained yeast-peptone-dextrose (YPD) or synthetic dextrose plates. Methyl methanesulfonate (MMS), 100 rad5-13,112 his3-1 ade2-1 can1-100 rad5-535, and BPS were from Sigma. The antibiotic carbenicillin (100 μg/ml) was in the growth medium. Chloramphenicol, 10 μg/ml, and tetracycline, 5 μg/ml, were used to select plasmid- and integration-derived clones, respectively. MOPS, 4-morpholinepropanesulfonic acid; BPS, bathophenanthroline disulfonic acid; MMS, methyl methanesulfonate.

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Plasmids—The plasmid pJM63, encoding HEM1, was prepared by amplifying the entire HEM1 gene using the primers HEM1-300F-Bam and HEM1+1722R-Eco and subcloning the product into the plasmid YEplac195. Similarly, HEM2 was amplified with the primers HEM2-300F-Hind and HEM2+1028R-Eco and subcloned into YEplac195, forming the plasmid pJM64. For NSGI overexpression, the entire NSGI gene was amplified using the primers NSGI-300F and NSGI+873R-Myc and was initially subcloned into the PCR cloning plasmid pCR2.1, forming the plasmid pJM76. The NSGI fragment was the liberated as a Xho1-BamHI fragment and subcloned into the SacI and BamHI sites of YEplac195, forming the plasmid pJM77.

FET3 Promoter Assays—The FET3-lacZ plasmid, consisting of the FET3 promoter fused to lacZ in the plasmid YEp354, was the kind gift of Dr. Jerry Kaplan and has been described previously (27). For lacZ measurement, cells were grown in synthetic medium, with or without 100 μM bathophenanthroline disulfonic acid (BPS) for 3 h. The A600 was measured, and the cells were centrifuged and lysed with 250 μl of the Y-PER solution (Pierce), and the lysate was incubated with 700 μl of Z buffer (60 mM Na2HPO4-7H2O, 40 mM NaH2PO4-H2O, 10 mM KCl, 1 mM MgSO4, and 50 mM β-mercaptoethanol) containing 1 mg/ml α-nitrophenyl β-D-galactopyranoside. The reaction was stopped with 500 μl of 1 M Na2CO3, clarified by centrifugation, and measured at 420 nm, using the same concentrations of Y-PER, Z buffer, and Na2CO3 as a blank. Miller units ((A420 × 1000)/(A600/min/ml)) were calculated for each point. FET3 transcription was assayed independently by reverse transcription-PCR as described previously (8) with the FET3 primers FET3+100F (ACAGGAACGTTGATGGGCTA) and FET3+380R (GAATGGTACCAGTAGGTGCC). Primers for the SCS2 transcript (8) served as a control for loading and were included in the same reaction as for FET3, and the products were separated in 2% agarose. Assays for ferric reductase activity were performed as described previously (28).

Protein Analysis—Log phase yeast cultures were lysed in Y-PER lysis solution (Pierce) containing 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml aprotinin and analyzed essentially as described (8). The antibody to HA (HA11) was from BabCo, and the antibody to α-tubulin was developed by J. Frankel and was obtained from the Developmental Studies Bank at the University of Iowa under the auspices of the NICHD, National Institutes of Health.

For cell fractionation, cell fractions were separated by sucrose gradient essentially as described (29). Briefly, 500 ml of log phase cells were arrested with the addition of 10 mM sodium azide, chilled on ice water, and then centrifuged at 4000 × g for 10 min. Cells were resuspended in spheroplasting buffer (1 M sorbitol, 100 mM Tris, pH 7.8, 10 mM EDTA, and 0.3 mg/ml zymolase) and incubated at 30°C for 40 min. Spheroplasts were

2 The abbreviations used are: YPD, yeast-peptone-dextrose; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; BPS, bathophenanthroline disulfonic acid; MMS, methyl methanesulfonate.
then centrifuged at 700 × g for 10 min and resuspended in 5 ml of lysis buffer (0.8 M sorbitol, 10 mM MOPS, pH 7.2, and 1 mM EGTA) containing 1 mM phenylmethylsulfonyl fluoride. The cells were then lysed with three 10-s pulses from a Polytron PT1200 homogenizer, and the homogenate was centrifuged at 2500 × g for 10 min to remove unlysed cells. One ml of the lysate was separated on a discontinuous 12–60% sucrose gradient by centrifugation at 100,000 × g for 16 h. at 4 °C. Fractions were collected and analyzed by Western blot using antibodies to the peroxisome marker Ypt7 (a kind gift from Dr. William Wickner), the mitochondrial marker Por1 (Molecular Probes), the lipid particle marker Erg6 (a kind gift from Dr. Gunther Daum), the endosomal marker Pep12 (Molecular Probes), the plasma membrane ATPase Pma1 (a kind gift from Dr. Ramon Serrano), and Erg11 (8).

Fluorescence—Staining was performed largely as described (29). Log phase diploid cells were fixed with 3.7% formaldehyde at 37 °C for 30 min, centrifuged, resuspended in 1 ml sorbitol containing 3.7% formaldehyde, and rotated at 4 °C overnight. Cells were spheroplasted in 1 M sorbitol containing 10 mM zymolase and 70 mM β-mercaptoethanol at 30 °C for 1 h, washed once in PBS, and applied to a poly-L-lysine-coated slide. Cells were then permeabilized with ice-cold methanol, blocked with phosphate-buffered saline containing 1 mg/ml bovine body (BabCo) for Dap1-HA and an anti-Myc tag antibody (Genscript) for Erg11-Myc, followed by fluorescein isothiocyanate conjugated secondary antibodies. For FM4–64 staining, nate-conjugated secondary antibodies. For FM4–64 staining, 5 μM FM4–64 (Molecular Probes) was added to the cells, which were imaged on a Zeiss Axioskop microscope. The cells were then fixed with 100% methanol, blocked with 10% FBS and incubated with an anti-HA antibody (H9004) and an anti-Myc tag antibody (H9004). The cells were then visualized with a Zeiss Axioskop microscope.

Sterol Analysis—200 ml of early log phase cells were grown in YPD medium and treated with 100 μM BPS for 3 h. Cells were pelleted and washed once with distilled water and extracted with potassium hydroxide-ethanol. Sterols were subsequently extracted with hexane, as described previously (4, 8), and analyzed by gas chromatography at the University of Kentucky Mass Spectrometry Facility.

RESULTS

Strains Lacking Dap1 Are Sensitive to Iron Depletion—Wild-type and dap1Δ strains were maintained on iron-depleted medium by culturing in 100 μM BPS. Wild-type cells grew normally, whereas the dap1Δ strains did not (Fig. 1A, lanes 3 and 4). A small zone of residual growth in the dap1Δ strain was dark red and consisted of nonbudded cells with a disrupted morphology. The BPS sensitivity of dap1Δ cells was complemented by the wild-type DAPI1 gene (Fig. 1B, row 7) but not by the DAPI1-D91G mutant (Fig. 1B, row 8), which does not bind to heme (8). The results suggest that the heme binding function of Dap1 is required for growth under iron-depleted conditions.

The expression of the multicopper oxidase Fet3 is induced under iron-depleted conditions (30). A construct containing the FET3 promoter fused to the bacterial lacZ gene was used to measure FET3 expression. FET3 increased 22-fold in wild-type cells and 31-fold in dap1Δ cells following iron depletion (Fig. 1C), a difference that was statistically significant (p = 5 × 10^{-6}, two-tailed t test). The difference in FET3 transcription in BPS-treated wild-type and dap1Δ cells was confirmed using using reverse transcription-PCR, where we reproducibly detected a 25% increase in FET3 levels (Fig. 1D). Despite increased FET3 transcription, we did not detect any change in the uptake of radiolabeled iron or iron reductase activity (50.5 ± 2.8 nmol of Fe2+/min/A660 for wild type versus 52.6 ± 5.0 for dap1Δ). The results suggest a disparity between iron-regulated transcription and iron uptake in dap1Δ cells.

Because Dap1 regulates sterol synthesis, we tested the effect of the Dap1 mutation on sterol biosynthesis under low iron conditions. As expected, dap1Δ cells had elevated lanosterol and episterol relative to wild-type cells (Fig. 2, compare A and C). Treatment of wild-type cells with BPS caused a modest accumulation of episterol (Fig. 2B), whereas dap1Δ cells accumulated increased levels of squalene (Fig. 2D), suggesting an iron-related defect in Erg1 function in dap1Δ cells. The sterol synthetic pathway is diagrammed in Fig. 2E. Although the Erg1 substrate was elevated under iron-depleted conditions, multi-copy expression of Erg1 did not suppress BPS sensitivity in dap1Δ cells (Fig. 2F, bottom, compare bottom rows), indicating that altered Erg1 function is not directly related to iron metabolic defects in dap1Δ cells.

**Erg11 Suppresses the Requirement for Dap1 in Iron Metabolism**—Because Dap1 requires heme binding for sterol synthesis, damage resistance, and viability in low iron, we devel-

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**FIGURE 1. Dap1 is required for growth under iron-depleted conditions.** A, wild-type (RCY409-2a) or dap1Δ (RCY409-4b) cells were serially diluted 1:10 and tested for viability on normal growth medium (rows 1 and 2) or the same medium containing 100 μM BPS, an iron-chelating agent. The dap1Δ strain grew poorly, and the residual colonies exhibited a dark red color. B, wild-type cells harboring a control vector (rows 1 and 5) or dap1Δ cells harboring the control plasmid pRS313 (rows 2 and 6), the DAPI1 expression plasmid pRC41 (rows 3 and 7), or the DAPI1-D91G expression plasmid pRC39 (rows 4 and 8) were tested for growth on control medium (rows 1–4) or medium containing BPS (rows 5–8). DAPI1-D91G encodes a point mutant of Dap1 that is incapable of binding to heme. The wild-type DAPI1 gene complemented the dap1Δ mutation, whereas the DAPI1-D91G mutant did not, demonstrating a requirement for heme binding for growth on BPS. C, Dap1 suppresses the expression of iron-regulated genes. Wild-type or dap1Δ cells harboring a FET3-lacZ construct were untreated (light gray columns) or were treated with 100 μM BPS (dark gray columns), and β-galactosidase activity was determined in triplicate. Error bars, the S.D. between individual measurements. D, FET3 transcripts analyzed by reverse transcription-PCR in which wild-type cells (lanes 2 and 3) or dap1Δ cells (lanes 4 and 5) were untreated (lanes 2 and 4) or treated with 100 μM BPS (lanes 3 and 5) for 3 h. Primers directed to the SC52 transcript, which is not regulated by iron, were included as an internal control for cDNA loading (lower band, labeled CON for “control”), and lane 1 is a negative control in which the RNA template from the sample in lane 2 was amplified without added reverse transcriptase. The numbers below each lane indicate the FET3/control ratio relative to the untreated wild-type cells in lane 2. For each assay, the results shown represent experiments that were performed at least in triplicate.

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**FIGURE 2. Dap1 regulates sterol metabolism.**
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opoped a genetic system for increasing heme levels. The HEM1/δ-aminolevulinate synthase and HEM2/δ-aminolevulinate dehydratase genes were expressed at high copy numbers using the multiple copy plasmid Yeplac195, and this suppressed the elevated susceptibility of dap1Δ cells to the alkylating agent methyl methanesulfonate (MMS) (Fig. 3A). The effect was more pronounced for HEM2 than HEM1 (Fig. 3A, rows 11 and 12). However, HEM1 and HEM2 high copy expression did not suppress itraconazole sensitivity, suggesting that the expression of these genes is not limiting for sterol synthesis (Fig. 3A, rows 17 and 18). Nsg1 is the yeast homologue of the human Insig-1 protein, which binds to the human Dap1 homologue (31). However, a high dosage of NSG1 did not suppress MMS, itraconazole, or BPS susceptibility in dap1Δ cells (data not shown).

Although the heme-binding domain of Dap1 was required for MMS resistance, high dose expression of HEM2 did not suppress BPS sensitivity in dap1Δ cells (Fig. 3B, row 11). In contrast, high dosage of ERG11 completely suppressed BPS sensitivity (Fig. 3B, row 12). This effect was not generalized to other sterol biosynthetic genes, because high dosage of ERG1 and ERG5 had no effect on BPS sensitivity in dap1Δ strains (data not shown). Dap1 binds to heme and is related to cytochrome b₅, encoded by CYB5 in S. cerevisiae, but high copy expression of CYB5 did not suppress loss of Dap1 (data not shown).

Because ERG11 suppressed loss of DAP1, we tested the extent to which Erg11 inhibitors affected growth on iron-depleted medium. We detected partial growth of wild-type cells on 2 μM itraconazole and 100 μM BPS (Fig. 3C, rows 2 and 3, respectively) but essentially no growth when the two compounds were combined (Fig. 3C, row 4). Taken together, the results suggest that sterol synthesis mediated by Erg11 and Dap1 is required for survival in iron-depleted medium, and this Dap1 function is not a general property of cytochrome-like proteins.

Characterization of Dap1—We fused three copies of the HA epitope tag sequence through one-step integration to the 3' end of the DAP1 gene. As a result, DAP1 was transcribed from its own promoter and synthesized from a single copy of its gene. The tagged Dap1 protein was readily detected as a 25-kDa protein by Western blot (Fig. 4A, top, lanes 2 and 3). In contrast, strains lacking the in frame epitope tag did not have a detectable 27-kDa protein (Fig. 4A, top, lane 1). Thus, the migration of Dap1 was similar to that of related rat and human proteins. In all cases, blots were probed with an antibody to tubulin as a control for protein loading (Fig. 4A, bottom).

Dap1 expression was reported previously to change upon treatment with the antifungal triazole drug itraconazole (7), and dap1Δ mutants are sensitive to itraconazole (4, 8). The expression of Dap1 increased 14-fold in cells treated with 0.1–1 μM itraconazole in a dose-dependent manner (Fig. 4B). In contrast, Dap1 expression did not change significantly following treatment with MMS, heat shock, or hydroxyurea (supplemental Fig. 2A). In addition, we did not detect any changes in Dap1 expression following treatment with BPS (supplemental Fig. 2B) or in strains with elongated or shortened telomeres (tel1Δ or rif1Δ rif2Δ, respectively; supplemental Fig. 2C) or in strains with acute damage sensitivity (mecl-21, rad9Δ, or dun1Δ).

Dap1 Localizes to Punctate Cytoplasmic Sites and Co-frac-tionates with Endosomal Markers—A diploid strain expressing the tagged Dap1 protein (Fig. 4A, lane 4), which was not expressed in the control untagged diploid strain (Fig. 4A, top, lane 5) was stained by immunofluorescence with the HA antibody. Dap1 localized to bright, clearly defined spots within the cytoplasm (Fig. 4C; bright field in Fig. 4D). The spots varied in size and number between the cells and were detectable in both mother and daughter cells. There was no evidence for Dap1 staining within the mother-bud neck, within the nucleus, or at the cell periphery. In contrast, the control diploid strain did not stain with the HA antibody after the same procedure (Fig. 4E; bright field shown in Fig. 4F). Similar spots of Dap1 staining were detected in haploid strains but were more difficult to visualize due to the smaller cell size.

Punctate cytoplasmic staining is characteristic of several types of subcellular structures. Thus, the subcellular fractions of a Dap1-tagged strain were separated on a sucrose gradient...
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and probed for Dap1 or various markers. Dap1 co-fractionated with Ypt7 (Fig. 5, A and B), a GTP-binding protein that regulatesvacular transport (32), indicating that Dap1 co-localizes with endosomal proteins. Dap1 also partially co-fractionated with Pep12, a Qa+ type SNARE/syntaxin (33) that is a marker for late endosomes (Fig. 5C). In contrast, Dap1 did not co-fractionate with Erg6/sterol C-24 methyltransferase, a marker for lipid particles (Fig. 5D) (34, 35) or with Por1/porin, a voltage-gated anion channel marker for mitochondria (Fig. 5E). The finding that Dap1 did not co-fractionate with Erg6 is consistent with an earlier mass spectrometric analysis of abundant proteins in purified lipid particles, which did not identify Dap1 (34, 36).

Mass spectrometric screens for interacting proteins have identified the plasma membrane ATPase Pma1 as a putative Dap1-binding protein (37). However, our gradient detected Pma1 in a distinct portion of the gradient from Dap1 (Fig. 5F). Dap1 interacts genetically with Erg11 and stabilizes Erg11 expression (8). Erg11 localization was diffuse throughout the sucrose gradient (Fig. 5G), and Erg11 staining by immunofluorescence was granular and excluded from the vacuole (Fig. 5H). Previous studies with GFP-tagged Erg11 derivatives localized Erg11 to the endoplasmic reticulum (38), cytoplasm (35), or granular sites (39).

Ypt7 is related to the mammalian Rab7 protein and regulates the movement of proteins from the prevacuolar compartment to the vacuole (32). We tested the role of Dap1 in vacuolar structure by incubating wild-type and dap1Δ cells with the fluorescent dye FM4-64, which is internalized and stains the yeast vacuole (40). In wild-type cells, vacuoles stained as circular structures (Fig. 6C; the bright field image is shown in Fig. 6A), whereas in dap1Δ cells, a significant fraction of vacuoles consisted of multiple smaller structures that resembled unfused vesicles (Fig. 6D), and others contained irregular edges or a central septum. The color version of this figure is supplemental Fig. 3. The percentage of cells with aberrant vacuoles was elevated in dap1Δ cells (23 ± 5%) compared with wild type (7 ± 3%) to a significant extent (p = 0.006, two-tailed t test).

Defective vacuolar structure could inhibit vacuolar iron storage. In wild-type cells harboring a CCC1 (cross-complements \( \text{Ca}^{2+} \) phenotype of \( \text{cs} \) (41); \( \text{cs} \) is an abbreviation for calcium-sensitive growth) high copy plasmid, \( \text{FET3-lacZ} \) reporter activity was highly induced (Fig. 6E), most likely due to iron being sequestered in the vacuole. In dap1Δ cells harboring the same CCC1 plasmid, \( \text{FET3-lacZ} \) activity was diminished by 40% (Fig. 6E), a significant difference (p = 0.011, two-tailed t test). Decreased \( \text{FET3} \) transcription in \( \text{CCC1} \)-expressing dap1Δ cells was almost identical when \( \text{FET3} \) transcripts were assayed using an independent PCR technique (Fig. 6F, compare lanes 3 and 5). We detected a 38% decrease in \( \text{FET3} \) transcripts in dap1Δ cells expressing \( \text{CCC1} \) compared with comparable wild-type cells when calculated from the \( \text{FET3} \)/control transcript ratios (shown at the bottom of Fig. 6F). In triplicate analyses, the difference in \( \text{FET3} \) levels between \( \text{CCC1} \)-overexpressing wild-type and dap1Δ cells was statistically significant (p = 0.02, two-tailed t test). This finding is consistent with a diminished ability of \( \text{CCC1} \) to direct iron storage in dap1Δ cells. We note that the \( \text{CCC1} \) high copy plasmid did not affect growth of dap1Δ cells under low iron conditions, consistent with an additional defect in iron uptake.

DISCUSSION

Iron utilization in fungi is important clinically because the availability of free iron limits the growth of pathogenic yeast. In dap1Δ cells depleted of iron, \( \text{ERG11} \) activity is inhibited, causing toxic levels of lanosterol to accumulate. This effect is reversed by \( \text{ERG11} \) overexpression but not by elevated heme. It is unclear why iron depletion is toxic to cells containing elevated levels of lanosterol. One potential explanation is that the pathway that detoxifies lanosterol requires iron. Thus, deletion of \( \text{DAP1} \) increases lanosterol levels, and iron depletion increases its toxicity (diagrammed in Fig. 7). This model is supported by our finding that BPS increases susceptibility to azole drugs and by similar findings in \( \text{C. albicans} \) (42).

In cells lacking Dap1, iron deprivation triggers increased induction of \( \text{FET3} \) expression, but increased \( \text{FET3} \) levels do not lead to increased iron uptake or iron reductase activity, suggesting that the sterol imbalance in dap1Δ cells interferes with iron uptake. The model that sterol synthesis promotes iron metabolism is consistent with the findings of Li and Kaplan, in which
a mutation in ERG25/sterol C-5 methylxoxidase causes sensitivity to low iron (43). However, it is unlikely that dap1/H9004 cells are sensitive to low iron solely because of low levels of ergosterol. Because conditions that have a modest effect on ergosterol levels (i.e. overexpression of ERG11) can suppress low iron sensitivity in dap1/H9004 mutants, it is likely that the balance of sterol intermediates affects iron metabolism rather than ergosterol levels per se.

Deletion of DAP1 causes accumulation of the sterol intermediates lanosterol (the Erg11 substrate) and episterol (the Erg3 substrate) and of the sterol ergosta-5,7-dienol (4). Lanosterol accumulation can be suppressed by overexpressing ERG11, which restoresazole resistance to dap1Δ mutants and triggers a marked accumulation of episterol and ergosta-5,7-dienol (8). Episterol presumably accumulates because Dap1 is required for the activity of Erg3/sterol C-5 desaturase (Fig. 7) (44), which is a highly conserved protein that is activated by cytochrome b₅ (6, 45), with which Dap1 shares structural homology and heme binding activity. In addition, Erg3 is also proposed to bind to iron through a series of coordinated histidine residues (43, 46).
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Iron is imported into the cell and is incorporated into heme, among other molecules. Dap1 binds to heme from its endosomal locale and activates Erg11 and Erg3, promoting sterol synthesis. Erg11 catalyzes the demethylation of lanosterol, an essential step in ergosterol synthesis, and some of the downstream metabolites of Erg11 are also detected in endosomes. Erg1 is also activated by Dap1 under low iron conditions. Our results do not exclude the possibility that iron plays a role in detoxifying sterol intermediates that accumulate in cells lacking wild-type Erg11 function (dashed line, left). There are a number of regulatory interactions between heme, the sterol pathway, and the uptake of iron, and these are not indicated.

Under low iron conditions, dap1Δ cells accumulate squalene, suggesting a defect in Erg1/squalene epoxidase. Erg1 is an oxygen-requiring enzyme that is inhibited by the allylamine class of antifungal drugs (1). Although squalene was one of the primary sterols to accumulate in iron-deprived dap1Δ cells, overexpression of ERG1 did not restore viability to dap1Δ cells under low iron conditions, suggesting that defective Erg1 activity is not solely responsible for their defective growth.

The common target of Dap1 and its homologues is P450 protein activation, and this activity is shared among the S. cerevisiae, S. pombe (9), and rodent (12) Dap1 homologues. Mammalian Dap1 homologues are highly expressed in the liver, kidney, and adrenal, which are sites of P450 activity (10, 47, 48), and mammalian Dap1 homologues localize to microsomal fractions, including the endoplasmic reticulum (49, 50), which are also common sites for P450 proteins. The exception to this rule is in neuronal cells, where some mammalian Dap1 homologues also localize to the cell membrane (51, 52). Both the S. pombe Dap1 and human PGRMC1 bind directly to Cyp51 and other P450 proteins (9). In contrast, we have not detected a stable interaction between Dap1 and Erg11 in S. cerevisiae, and our analysis suggests only a partial overlap in their localization. Furthermore, Dap1-Erg11 complexes have not been detected using genome-wide proteomic screens or two-hybrid analyses. S. cerevisiae Dap1 differs from its mammalian and S. pombe homologues in that it lacks a membrane-spanning sequence, and it is possible that this sequence is required for a stable interaction with Erg11.

Based on the model proposed above, Dap1 probably regulates vacuolar structure via sterol synthesis. Ergosterol is nec-
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essay for vacuole formation (53–56), and yeast microsomes contain a significant fraction of the sterols fecosterol, episterol, and ergosta-5,7,9(11),22-tetraenol (57–59). Our results suggest a model in which the apparent vacuolar defects in dap1Δ cells limit the extent to which CCC1 sequesters iron in the vacuole. CCC1 is postulated to act as an iron transporter, and CCC1 overexpression triggers increased cellular iron uptake (24) and vacuolar iron storage (60). These functions are partially dependent on Dap1, although the mechanism linking these proteins is unknown.

The localization of Dap1 to intracellular vesicles and the broad distribution of Erg11 suggest that some steps in P450 activation and sterol synthesis are distributed throughout the cell. Indeed, green fluorescent protein-tagged forms of Erg2, Erg3, and Erg25 all localize to a vesicle fraction (35). Furthermore, Dap1 has been previously implicated in endocytosis (4), and the localization of Dap1 to an endosomal fraction suggests a role in intracellular transport. Although most sterol-defective mutants are hyperpermeable to dyes (61), dap1Δ mutants have defective dye uptake, particularly at low temperatures (4), suggesting that Dap1 is required for the endocytosis of some compounds (4). In addition, dap1Δ cells do not display abnormalities in membrane integrity (4) that are typical of erg mutants (61). The model in Fig. 7 is based on the assumption that the primary role of Dap1 is in sterol synthesis, although it is possible that Dap1 contributes to endosome function or vacuole structure through other, uncharacterized mechanisms.

Although heme is required for sterol synthesis, our results suggest that heme levels are not limiting for azole drug resistance but are limiting for MMS resistance. In contrast, we found that iron levels are limiting for azole drug resistance. Although the presumed target for MMS is DNA, Lum et al. (62) previously identified HEM1 and HEM2 in a screen for MMS targets using a genome-wide pool of heterozygous deletion strains. One interpretation of these findings is that MMS targets Hem1 and/or Hem2 directly. However, MMS sensitivity in HEM1/ hem1 and HEM2/hem2 strains could also arise from diminished heme levels due to hapolinsufficiency of 5-aminolevulinate synthase and δ-aminolevulinate dehydratase, respectively. The results are important, because MMS closely resembles chemotherapeutic drugs that are used clinically, and our results suggest a role for heme biosynthesis in susceptibility to chemotherapeutic drugs. Indeed, the human Dap1 homologue Hpr6/PGRMC1 is overexpressed in clinical tumors and cancer cell lines (50), and inhibition of Hpr6/PGRMC1 causes increased susceptibility to chemotherapeutic drugs (13).

Iron deficiency is the most prevalent nutritional disorder in the world, affecting almost two billion people (63), and abnormal iron metabolism contributes to a number of diseases, including Friedrich’s ataxia, hemochromatosis, aging, and microbial infections (64). Dap1 has homologues in pathogenic fungi, including Candida species, and our results suggest that Dap1 homologues may have a role in iron metabolism in those organisms. If so, Dap1 homologues may be useful as targets for therapeutics, particularly in combination with other antifungal agents or under conditions leading to deprivation of available iron. There are also Dap1 homologues in higher eukaryotic species, including mammals (10, 47, 65), and the rodent and human Dap1 homologues bind to heme (12). However, the role of these proteins in iron metabolism has not been examined.

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