

# A Novel Sterol-Signaling Pathway Governs Azole Antifungal Drug Resistance and Hypoxic Gene Repression in *Saccharomyces cerevisiae*

Nina D. Serratore,\* Kortany M. Baker,\* Lauren A. Macadlo,\* Abigail R. Gress,\* Brendan L. Powers,\* Nadia Atallah,<sup>†</sup> Kirsten M. Westerhouse,\* Mark C. Hall,\*<sup>†</sup> Vikki M. Weake,\*<sup>†</sup> and Scott D. Briggs\*<sup>†,1</sup>

\*Department of Biochemistry, and <sup>†</sup>Purdue University Center for Cancer Research, Purdue University, West Lafayette, Indiana 47907

ORCID IDs: 0000-0001-6429-2506 (M.C.H.); 0000-0002-5933-9952 (V.M.W.); 0000-0003-2852-3594 (S.D.B.)

**ABSTRACT** During antifungal drug treatment and hypoxia, genetic and epigenetic changes occur to maintain sterol homeostasis and cellular function. In this study, we show that SET domain-containing epigenetic factors govern drug efficacy to the medically relevant azole class of antifungal drugs. Upon this discovery, we determined that *Set4* is induced when *Saccharomyces cerevisiae* are treated with azole drugs or grown under hypoxic conditions; two conditions that deplete cellular ergosterol and increase sterol precursors. Interestingly, *Set4* induction is controlled by the sterol-sensing transcription factors, *Upc2* and *Ecm22*. To determine the role of *Set4* on gene expression under hypoxic conditions, we performed RNA-sequencing analysis and showed that *Set4* is required for global changes in gene expression. Specifically, loss of *Set4* led to an upregulation of nearly all ergosterol genes, including *ERG11* and *ERG3*, suggesting that *Set4* functions in gene repression. Furthermore, mass spectrometry analysis revealed that *Set4* interacts with the hypoxic-specific transcriptional repressor, *Hap1*, where this interaction is necessary for *Set4* recruitment to ergosterol gene promoters under hypoxia. Finally, an *erg3Δ* strain, which produces precursor sterols but lacks ergosterol, expresses *Set4* under untreated aerobic conditions. Together, our data suggest that sterol precursors are needed for *Set4* induction through an *Upc2*-mediated mechanism. Overall, this new sterol-signaling pathway governs azole antifungal drug resistance and mediates repression of sterol genes under hypoxic conditions.

**KEYWORDS** chromatin; *SET4*; hypoxia; antifungal drugs; epigenetics; sterol; gene expression; *Saccharomyces cerevisiae*

**S**TEROLS are a major class of neutral lipids that affect important physical properties of membranes in eukaryotic cells, such as membrane fluidity, transport, and permeability (Parks and Casey 1995; Espenshade and Hughes 2007). The primary sterol found in the cell membranes of all fungi and protozoa is ergosterol (Espenshade and Hughes 2007; Weete *et al.* 2010; Dupont *et al.* 2012). Ergosterol is the yeast equivalent to cholesterol in humans and it has important structural and signaling functions necessary for the survival of the

organism (Parks and Casey 1995; Mouritsen and Zuckermann 2004; Espenshade and Hughes 2007). In addition, ergosterol and cholesterol biosynthesis is of fundamental and medical interest because this pathway contains the targets of several antifungal and statin drugs (Parks and Casey 1995; Odds *et al.* 2003; Kathiravan *et al.* 2012).

In *Saccharomyces cerevisiae* and other yeast species, ergosterol biosynthesis is an oxygen-dependent pathway (Parks and Casey 1995; Kwast *et al.* 1998; Rosenfeld and Beauvoit 2003; Espenshade and Hughes 2007; Ishtar Snoek and Yde Steensma 2007). Under hypoxic or anaerobic conditions, ergosterol production is blocked, leading to its depletion (Rosenfeld and Beauvoit 2003; Joshua and Höfken 2017). Yeast respond to ergosterol depletion by signaling structural changes in the cell wall to facilitate uptake of exogenous ergosterol (Abramova *et al.* 2001; Alimardani *et al.* 2004; Ishtar Snoek and Yde Steensma 2007; Zavrel *et al.* 2013).

Copyright © 2018 by the Genetics Society of America  
doi: <https://doi.org/10.1534/genetics.117.300554>

Manuscript received August 21, 2017; accepted for publication December 19, 2017; published Early Online December 20, 2017.

Supplemental material is available online at [www.genetics.org/lookup/suppl/doi:10.1534/genetics.117.300554/-/DC1](http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.117.300554/-/DC1).

<sup>1</sup>Corresponding author: Department of Biochemistry, Purdue University, Hansen Life Science Research Bldg., 201 S. University St., West Lafayette, IN 47907. E-mail: [sdbriggs@purdue.edu](mailto:sdbriggs@purdue.edu)

Therefore, ergosterol homeostasis is a balance between ergosterol synthesis and sterol uptake from the exogenous environment.

To maintain ergosterol homeostasis, ergosterol (*ERG*) genes, which encode enzymes needed for ergosterol biosynthesis, are tightly controlled by transcription factors (Kwast *et al.* 2002; Davies *et al.* 2005; Davies and Rine 2006; Hickman and Winston 2007; Ishtar Snoek and Yde Steensma 2007; Joshua and Höfken 2017). Three transcription factors that are known to regulate *ERG* genes include *Hap1*, *Ecm22*, and *Upc2* (Fytlovich *et al.* 1993; Vik and Rine 2001; Becerra *et al.* 2002; Ter Linde and Steensma 2002; Tamura *et al.* 2004; Davies *et al.* 2005; Davies and Rine 2006). *Upc2* and *Ecm22* are paralogs and activate genes in response to sterol levels. During sterol depletion using statin or antifungal azole drugs, *ERG* genes are induced by *Upc2* and *Ecm22* (Vik and Rine 2001; Wilcox *et al.* 2002; Davies *et al.* 2005; Davies and Rine 2006; Joshua and Höfken 2017).

Under aerobic conditions, *Hap1* is a heme-dependent transcription factor that is required for the proper expression of mitochondrial respiratory and oxidative stress genes (Zhang and Hach 1999; Becerra *et al.* 2002). *Hap1* is also needed for steady state *ERG* transcript levels and full induction of *ERG* genes when cells are treated with lovastatin (Fytlovich *et al.* 1993; Becerra *et al.* 2002; Tamura *et al.* 2004; Davies and Rine 2006). Under hypoxic conditions, *Hap1* is a heme-independent transcription factor (Zhang and Hach 1999); however, depending on the strain, *Hap1* acts as a transcriptional repressor or activator. In a S288C FY strain that expresses an integrated wild-type (WT) copy of *HAP1*, *Hap1* functions together with the corepressor complex, *Tup1*, and *Cyc8* to repress *ERG* genes (Hickman and Winston 2007). In addition, deletion of *HAP1* resulted in an increase in *ERG* genes and other genes involved in ergosterol metabolism (Hickman and Winston 2007). In contrast, in a W303 strain that expresses an endogenous *HAP1*, *ERG* genes increase in expression when switched to hypoxic conditions (Davies and Rine 2006); although it was not determined if *Hap1* mediated this increase. Nevertheless, an aGH1 strain lacking *HAP1* showed a subset of genes were downregulated under hypoxia, suggesting *Hap1* can act as a transcriptional activator (Lombardia *et al.* 2000). Currently, it is unclear what mediates these strain-specific differences.

Although the aforementioned transcription factors are necessary for regulating ergosterol biosynthesis, additional studies are needed to determine how this medically relevant pathway is regulated and how sterol-sensing transcription factors function with epigenetic regulators. Epigenetic regulators have been shown to mediate yeast growth under antifungal drug treatment. For example, deletions of *BRE1*, *BRE2*, and *HOS2* results in a hypersensitive growth defect to the antifungal metabolite Brefeldin A (BFA) (Muren *et al.* 2001; South *et al.* 2013). Work from our laboratory demonstrated that loss of the H3K4 methyltransferase, *Set1*, or loss of the *Set1* complex members that affect H3K4 methylation, has a hypersensitive growth defect in the presence of BFA (South

*et al.* 2013). Interestingly, the BFA hypersensitivity observed in a *set1Δ* strain is due to decreased expression of conserved *ERG* genes resulting in decreased ergosterol production (South *et al.* 2013). Nevertheless, *set1Δ* strains become resistant to BFA when grown in the presence of ergosterol, which is due to the induction of sterol transporters and uptake of exogenously provided ergosterol (South *et al.* 2013). Although loss of *SET3* resulted in BFA hypersensitivity and decreased expression of *ERG* genes, *Set4*, the *Set3* paralog, was not investigated (South *et al.* 2013). Until this study, little was known about the biological and biochemical function of *Set4*.

In this study, we investigated the role of SET domain-containing epigenetic factors, in particular *Set4*, under conditions that alter ergosterol levels such as azole drug treatment and hypoxia. Initially, we determined that deletion of *SET1* and *SET3* results in hypersensitivity to the medically relevant antifungal drugs ketoconazole and fluconazole. In contrast, a deletion of *SET4* results in azole drug resistance, indicating that SET domain proteins govern antifungal drug efficacy. However, we demonstrate that the azole-resistant phenotype is independent of changes in *ERG11* or ABC transporter gene expression, mechanisms known to play a role in azole resistance. Interestingly, under ergosterol-limiting conditions, we show that *Set4* expression is induced by the sterol-sensing transcriptional activators *Upc2* and *Ecm22*. RNA-sequencing analysis determined that *Set4* is required for *ERG* gene repression. Importantly, we show that *Set4* directly targets the ergosterol gene promoters *ERG11* and *ERG3* under hypoxia and that *Set4* recruitment is dependent on the transcriptional repressor, *Hap1*. Finally, we demonstrate that *Set4* expression is upregulated in an *erg3Δ* strain, suggesting a precursor sterol, but not ergosterol, regulates *Set4* levels. Overall, we have discovered a new sterol–*Upc2* signaling pathway mediated by *Set4* that governs azole drug efficacy and sterol homeostasis under hypoxic conditions.

## Materials and Methods

### Plasmids and yeast strains

All plasmids and yeast strains are described in Supplemental Material, Table S1 and Table S2 in File S7. Note that, depending on the yeast strain, there are genomic differences in *HAP1*. Most *S. cerevisiae* strains including W303 strains contain a WT copy of *HAP1* (Davies and Rine 2006). Many, if not all, S288C strains including BY4741 and FY strains contain an in-frame Ty1 insertion near the 3' end of the *HAP1* gene (Gaisne *et al.* 1999; Hickman and Winston 2007). The *hap1-Ty1* gene fusion is expressed and replaces 13 amino acids of the *Hap1* C terminus as well as adds 32 amino acids from the Ty1 element (Gaisne *et al.* 1999). Because the *hap1-Ty1* gene fusion has been shown to partially compromise the function of *Hap1*, the FY2609 strain (the “*HAP1*-corrected” strain) was also used (Gaisne *et al.* 1999; Hickman and Winston 2007). FY2609 was modified by replacing the *hap1-Ty1* gene fusion with a WT copy of *HAP1* (Hickman and Winston 2007).

The yeast plasmids used in this study were constructed and PCR verified as previously described (South *et al.* 2010, 2013; Mersman *et al.* 2012; Zhang *et al.* 2016). FLAG-tagged strains were generated using the N-ICE plasmid tagging system and PCR verified (Zhang *et al.* 2016).

### **Yeast growth conditions**

Yeast growth and drug treatment were performed as previously described (Agarwal *et al.* 2003). The indicated strains were inoculated in SC media and grown to saturation overnight. Cells were diluted to an OD<sub>600</sub> of 0.1 and recovered to log phase for 3 hr shaking at 30°. Prior to treatment, cells were collected for the untreated sample and zero time point. Cultures were treated at an OD<sub>600</sub> of 0.2 with 56 µg/ml ketoconazole (Sigma-Aldrich, St. Louis, MO) dissolved in DMSO as previously described (Agarwal *et al.* 2003). Cells were collected every 3 hr. The indicated yeast strains were grown in YPD media to log phase under aerobic shaking conditions, or for 8 hr under hypoxia in a Bio-Bag Type A System (14-910-5; Fisher Scientific, Pittsburgh, PA). Cells were immediately pelleted and flash frozen.

### **Azole dilution assays and growth assays**

For dilution assays, yeast strains were inoculated in SC media and grown to saturation overnight. Yeast strains were diluted to an OD<sub>600</sub> of 0.1 and grown in SC media to log phase shaking at 30°. The indicated strains were spotted in fivefold dilutions starting at an OD<sub>600</sub> of 0.01 on untreated SC plates or plates containing 1 µg/ml ketoconazole (Sigma-Aldrich) or 12 µg/ml fluconazole (Sigma-Aldrich). Plates were grown at 30° for 2–5 days. For growth assays, the indicated yeast strains were inoculated in SC media and grown to saturation overnight. Yeast strains were diluted to an OD<sub>600</sub> of 0.1 and grown in SC media to log phase shaking at 30°. The indicated strains were diluted to an OD<sub>600</sub> of 0.0001 in 100 µl SC media. Cells were left untreated or treated with 20 µg/ml ketoconazole and grown for 50 hr shaking at 30°. The cell density OD<sub>600</sub> was determined every 2 hr using the Bio-Tek Synergy 4 multi-mode plate reader (<http://www.biotek.com>).

### **Yeast extraction and Western blot analysis**

Whole cell extraction and Western blot analysis to detect 3×FLAG-Set4, 3×FLAG-Set3, histone modifications, and glucose-6-phosphate dehydrogenase (G6PDH) were performed as previously described (Briggs *et al.* 2001; Fingerman *et al.* 2005; Mersman *et al.* 2012). The polyclonal anti-FLAG rabbit antibody was used to detect 3×FLAG-Set4 and 3×FLAG-Set3 expression at 1:5000 (catalog F7425; Sigma-Aldrich). The H3K4 methylation-specific and anti-G6PDH antibodies were used as previously described (South *et al.* 2013; Harmeyer *et al.* 2015).

### **Gene expression analysis**

RNA was isolated from cells by standard acid phenol purification. Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA)

per the manufacturer's instructions. Primer Express 3.0 software was used for designing primers (see Table S3 in File S7) and quantitative real-time polymerase chain reaction (qRT-PCR) was performed as previously described (South *et al.* 2013; Zhang *et al.* 2016). Three biological replicates, including three technical replicates, were performed for all samples. Data were analyzed using the  $\Delta\Delta C_t$  method where *ACT1* or *RDN18-1* [18S ribosomal RNA (rRNA)] was used as an internal control. All samples were normalized to an untreated, untagged WT strain. The unpaired *t*-test was used to determine the statistical significance between two strains at the genes of interest. All statistical values were reported as the raw *P*-values. The mean, SD, SEM, and statistical significance for appropriate data can be found in Table S5 in File S7.

### **Immunoprecipitation for mass spectrometry analysis**

For analysis of Set4 protein interaction partners, 200 ml of the 3×FLAG-Set4 and the untagged WT strains were grown to log phase under hypoxia for 8 hr. Whole cell lysate was prepared from harvested cells by lysing cells with glass beads and bead beating. Cells were lysed with 2 ml of lysis buffer (20 mM HEPES, pH 7.5, 350 mM NaCl, 10% glycerol, 0.1% Tween 20) containing protease and phosphatase inhibitors as previously described (Mersman *et al.* 2012). For immunoprecipitation, 60 µl of Protein G Magnetic Dynabeads were conjugated with 20 µl M2 FLAG antibody and immunoprecipitated with 2 ml of whole cell lysate for 2 hr rotating at 4°. Proteins were resolved on a 10% SDS-PAGE gel and whole lanes were cut out for in-gel trypsin digestion. For details on mass spectrometry (MS) analysis see File S4 and the supplemental methods in File S6.

### **Chromatin immunoprecipitation**

ZipChIP was performed as previously described (Harmeyer *et al.* 2015). Briefly, 50 ml cultures were grown to log phase (OD<sub>600</sub> of 0.6) in YPD media at 30° under aerobic shaking conditions or 8 hr of hypoxia. Cells were formaldehyde cross-linked and harvested as previously described (Harmeyer *et al.* 2015). Cell lysates were precleared with 5 µl of unbound Protein G magnetic beads for 30 min rotating at 4°. A total of 200 µl of precleared lysate was immunoprecipitated with 10 µl of Protein G magnetic beads (10004D; Life Technologies) conjugated to 1 µl of M2 FLAG antibody (F1804; Sigma-Aldrich) or MYC antibody (9E10). Probe sets used in qRT-PCR are described in Table S4 in File S7.

### **RNA sequencing**

The BY4741 WT and *set4*Δ strains were grown to log phase in aerobic and hypoxic conditions for 8 hr in YPD media. Total RNA of three biological replicates for each condition and sample were isolated by standard acid phenol purification, treated with DNase (Ambion), and total RNA was purified using standard acid phenol purification. The quality of the RNA was tested using an Agilent Bioanalyzer 2100 using the High Sensitivity DNA Chip. The complementary DNA library

was prepared by the Purdue Genomics Facility using the TruSeq Stranded Kit with poly(A) selection (Illumina) according to the manufacturer's instructions. For more details on RNA-sequencing analysis see the supplemental methods in File S6.

### Data availability

Strains are available upon request. File S5 and File S7 contain additional tables and figures including the strain list and genotypes, gene expression primers, and chromatin immunoprecipitation (ChIP) probe sets. File S1, File S2, File S3, and File S4 include gene expression data from the RNA-sequencing analysis and peptide hits from the MS analysis. Genome-wide RNA-sequencing data are available at Gene Expression Omnibus under accession number GSE107492.

## Results

### Set1 and Set3 govern azole drug sensitivity

Our previous work determined that loss of *Set1* or *Set3* resulted in hypersensitivity to BFA due to the decreased expression of the ergosterol gene, *ERG11*, which encodes the enzyme inhibited by azole antifungal drugs such as ketoconazole and fluconazole (Odds *et al.* 2003; South *et al.* 2013). To test the role of SET domain proteins in drug sensitivity, particularly *Set4*, we performed liquid growth assays in the presence and absence of the medically relevant azole drug, ketoconazole, using WT, *set1* $\Delta$ , *set3* $\Delta$ , and *set4* $\Delta$  strains. All strains grew similarly without the drug (Figure 1A), while *set1* $\Delta$  and *set3* $\Delta$  strains showed hypersensitivity toward 20  $\mu$ g/ml ketoconazole when compared to WT (Figure 1A) and similarly to what we observed for BFA (South *et al.* 2013). Interestingly, the *set4* $\Delta$  strain grew better than WT in the presence of ketoconazole (Figure 1A), indicating that *Set3* and *Set4* have different biological functions.

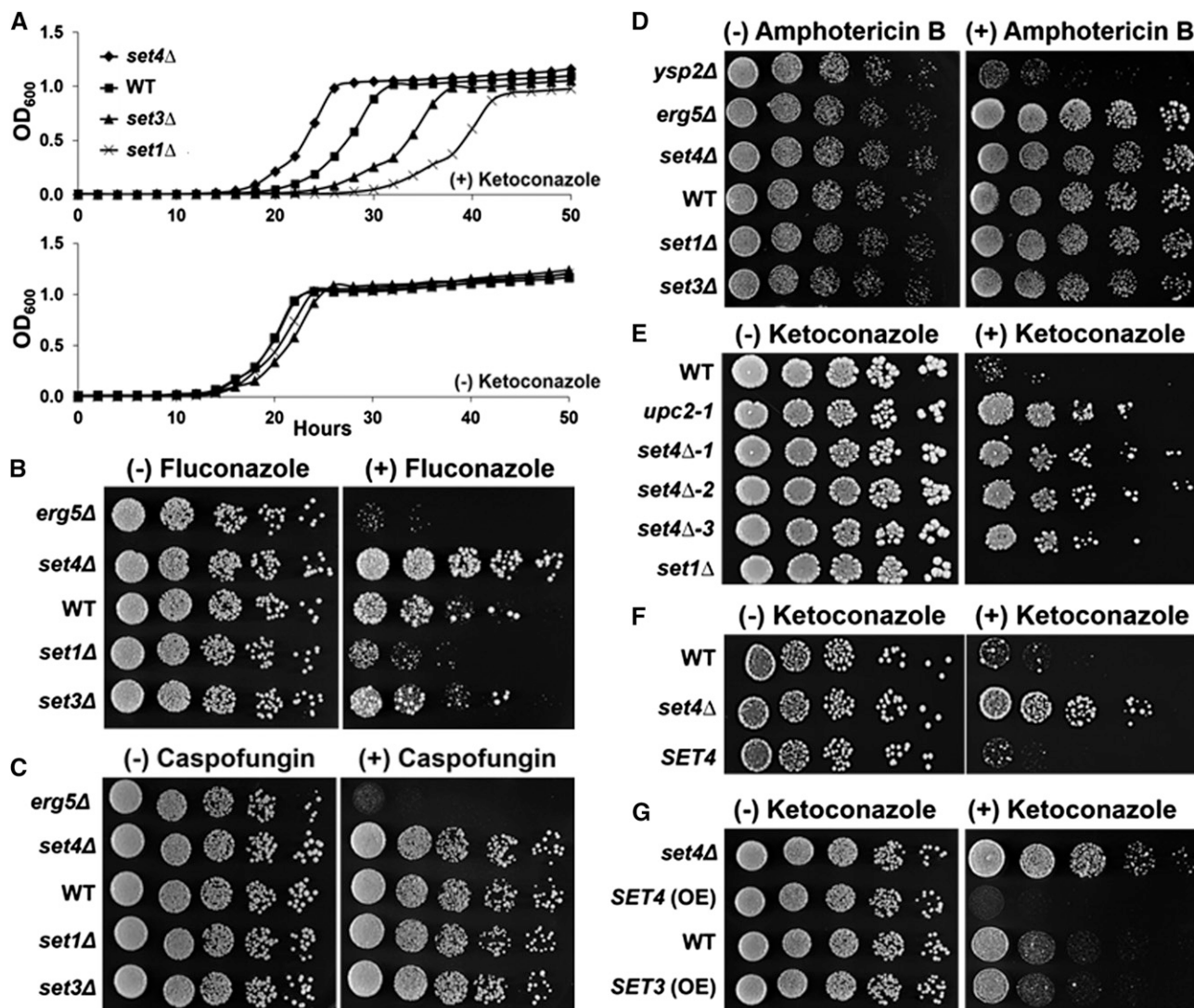
To determine if the two subclasses of azole drugs, imidazole and triazole, would behave similarly, we performed dilution assays on plates containing the triazole drug, fluconazole, using WT, *set1* $\Delta$ , *set3* $\Delta$ , and *set4* $\Delta$  strains. Similar to what was observed for liquid growth assays with ketoconazole, the *set1* $\Delta$  strain showed hypersensitivity when grown on plates containing 12  $\mu$ g/ml fluconazole (Figure 1, A and B), while the *set3* $\Delta$  strain did not have an apparent growth difference (Figure 1B). Intriguingly, the *set4* $\Delta$  strain grew better than WT on fluconazole plates, suggesting that the loss of *SET4* leads to azole drug resistance (Figure 1B). The DMSO-treated WT, *set1* $\Delta$ , *set3* $\Delta$ , and *set4* $\Delta$  strains grew similarly, demonstrating that the changes in observed growth are due to azole drug treatment (Figure 1, A and B). Additionally, the *erg5* $\Delta$  strain was used as positive control for fluconazole sensitivity. Altogether, these results demonstrate that SET domain proteins govern the efficacy of both the imidazole and triazole subclasses of azole antifungal drugs.

To characterize the extent of SET domain proteins in drug sensitivity and resistance, we tested whether *set1* $\Delta$ , *set3* $\Delta$ , and

*set4* $\Delta$  strains were sensitive to other classes of antifungal drugs including the echinocandin drug, caspofungin, and the polyene drug, amphotericin B (Odds *et al.* 2003; Campoy and Adrio 2017). Dilution assays were performed on plates containing 0.25  $\mu$ g/ml caspofungin or 0.5  $\mu$ g/ml amphotericin B. In contrast to the azole drug assays, the *set1* $\Delta$ , *set3* $\Delta$ , and *set4* $\Delta$  strains grew like WT on plates containing either caspofungin or amphotericin B; indicating that the phenotypes observed in the *set1* $\Delta$ , *set3* $\Delta$ , and *set4* $\Delta$  strains were specific to the azole class of antifungal drugs (Figure 1, C and D). The *erg5* $\Delta$  and *ysp2* $\Delta$  strains were used as positive controls for drug sensitivity (Markovich *et al.* 2004; Gatta *et al.* 2015). To further analyze the *set4* $\Delta$  strain, we tested whether the resistant-like growth observed in a *set4* $\Delta$  strain treated with azole drugs grows similarly to *upc2-1*, a well-known, azole-resistant mutant (Flowers *et al.* 2012). Dilution assays were performed on ketoconazole plates spotted with three independent knockouts of *SET4* and the *upc2-1* mutant strain. All three *set4* $\Delta$  strains grew like the *upc2-1* mutant and grew better than WT, providing strong evidence that yeast strains lacking *Set4* will consequently lead to azole drug resistance (Figure 1E). Again, all strains grew similarly on plates without the drug, showing that all strains were equally spotted, and the *set1* $\Delta$  strain was used as a control for azole sensitivity (Figure 1E).

To confirm that loss of *Set4* promotes azole drug resistance, we performed a rescue experiment by adding back *SET4* to the *set4* $\Delta$  strain and analyzing yeast growth in the presence and absence of ketoconazole using a dilution plate assay. The WT and *set4* $\Delta$  strains transformed with an empty yeast plasmid vector or *SET4* driven from the endogenous promoter were spotted on SC-Ura plates with or without 1  $\mu$ g/ml ketoconazole. The WT strain transformed with empty vector and the *set4* $\Delta$  strain transformed with endogenous *SET4* grew similarly on ketoconazole plates, whereas the *set4* $\Delta$  strain transformed with empty vector showed resistant growth on ketoconazole plates (Figure 1F). All three strains grew similarly on SC-Ura plates without the drug (Figure 1F).

Because a *set4* $\Delta$  strain is resistant to ketoconazole and fluconazole, we hypothesized that overexpression of *Set4* would lead to azole hypersensitivity. To test this hypothesis, we integrated a constitutive *PYK1* promoter upstream of *SET4* and *SET3* using our N-ICE plasmid system and performed a dilution assay on ketoconazole and fluconazole plates (Zhang *et al.* 2016). Overexpression of *Set4* resulted in hypersensitive growth compared to WT under ketoconazole or fluconazole treatment, suggesting that too much *Set4* negatively affects cell viability (Figure 1G). Overexpression of *Set3* grew similarly to WT, further demonstrating that *Set4* and *Set3* play distinct roles in governing azole efficacy (Figure 1G). The *set4* $\Delta$  strain was used as a control to show drug resistance (Figure 1G). All strains grew similarly on SC plates, indicating that overexpression of *Set4* or *Set3* did not alter growth conditions. To verify that *Set4* and *Set3* were overexpressed in the strains generated using the N-ICE plasmid tagging system, qRT-PCR and Western blot analysis were used to



**Figure 1** SET domain proteins govern azole antifungal drug sensitivity and resistance. (A) Growth curve of indicated BY4741 strains over a 50-hr time course in SC media with 20  $\mu\text{g/ml}$  ketoconazole or SC media. (B–E) Dilution assays of the indicated BY4741 strains spotted on SC plates containing 12  $\mu\text{g/ml}$  fluconazole, 0.5  $\mu\text{g/ml}$  caspofungin, 0.5  $\mu\text{g/ml}$  amphotericin B, or 1  $\mu\text{g/ml}$  ketoconazole. (F) Dilution assay of BY4741 WT or *set4* $\Delta$  strains transformed with plasmids containing *SET4* from its endogenous promoter or empty vector spotted on SC-Ura plates with 1  $\mu\text{g/ml}$  ketoconazole. (G) Dilution assay of BY4741 WT, *set4* $\Delta$ , and overexpressed *SET4* and *SET3* strains. Strains were spotted on SC plates with 1  $\mu\text{g/ml}$  ketoconazole.

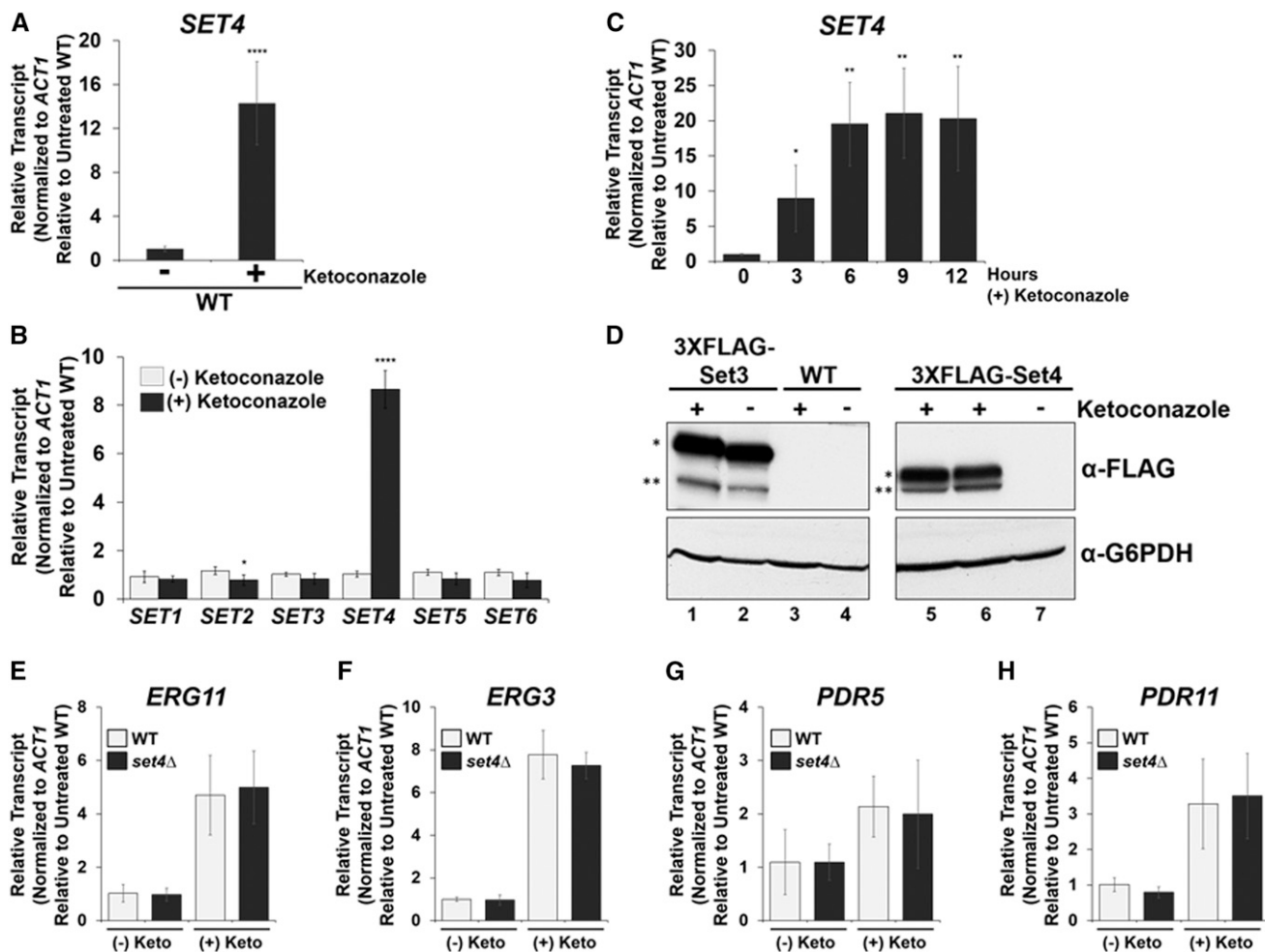
detect *Set4* and *Set3* expression levels (Figure S1, A and B, in File S5). As expected, *Set4* and *Set3* expression increased significantly in the overexpression strains compared to the endogenous tagged strains (Figure S1, A and B, in File S5). Overall, these results establish that the loss of *SET4* is responsible for azole resistance and that cellular levels of *Set4* are important for governing this phenotype.

#### ***Set4* expression is induced under azole antifungal drug treatment**

Because *Set4* levels are critical for altering azole resistance, endogenous *SET4* messenger RNA (mRNA) levels were quantified from WT cells that were treated with ketoconazole or DMSO by qRT-PCR analysis. Interestingly, *SET4* transcript levels increase significantly (16-fold) following 3 hr

of treatment with 56  $\mu\text{g/ml}$  ketoconazole (Figure 2A). Actin mRNA levels were used to normalize *SET4* transcript levels and the ketoconazole-treated WT sample was set relative to DMSO-treated WT cells. These results are consistent with a microarray study that showed increased *SET4* expression upon treatment with ketoconazole (Agarwal *et al.* 2003).

Because deletions of *SET1* and *SET3* result in sensitivity to azole treatment, we tested whether transcript levels of genes encoding SET domain proteins are affected by ketoconazole treatment. WT cells were treated with ketoconazole and qRT-PCR was used to analyze *SET1–SET6* expression. *SET1*, *SET3*, *SET5*, and *SET6* transcript levels were not significantly altered in WT cells treated with ketoconazole; however, *SET2* expression showed a 38% decrease in transcript levels under ketoconazole (Figure 2B). The *SET1–SET6* mRNA levels from

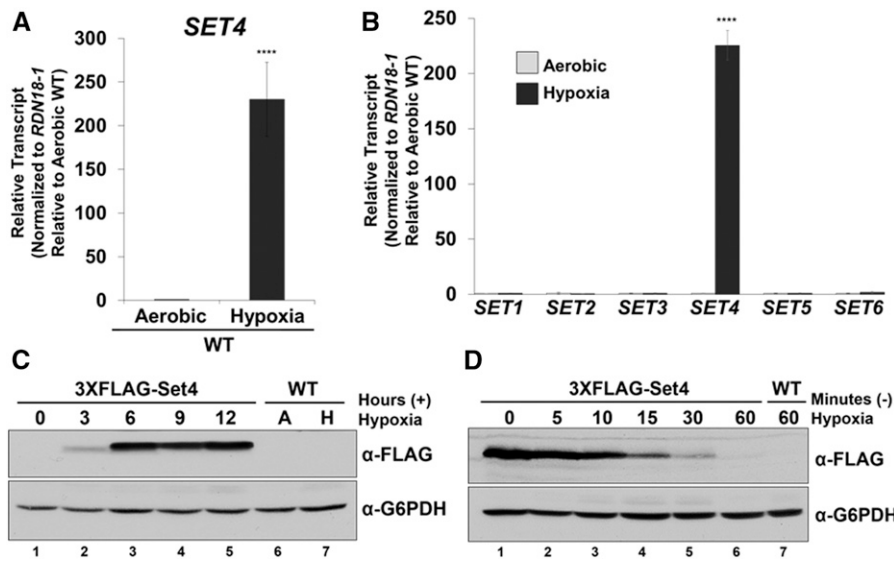


**Figure 2** Azole antifungal drug treatment induces Set4 expression. (A and B) Expression of indicated genes was determined in WT cells treated with DMSO or 56  $\mu\text{g/ml}$  ketoconazole for 3 hr by qRT-PCR analysis. Statistical analysis identified significant changes for *SET2* and *SET4* expression (\*  $P < 0.05$  and \*\*\*\*  $P < 0.0001$ , respectively). (C) *SET4* expression was determined in WT cells treated with DMSO or 56  $\mu\text{g/ml}$  ketoconazole by qRT-PCR analysis. Gene expression analysis was set relative to the DMSO-treated WT and expression was normalized to actin mRNA levels (*ACT1*). Data were analyzed from three biological replicates with three technical replicates. Error bars represent SD. \*  $P < 0.05$ , \*\*  $P < 0.01$ . (D) Western blot analysis of Set3 and Set4 protein levels under DMSO and 56  $\mu\text{g/ml}$  ketoconazole treatment. Lane 6 represents 3 hr of ketoconazole treatment. Lanes 1, 3, and 5 represent 6 hr of ketoconazole treatment. Lanes 2, 4, and 7 show untreated Set3, untagged WT, and Set4, respectively. G6PDH was used as a loading control. \* indicates 3XFLAG-Set3 and 3XFLAG-Set4 protein levels, respectively. \*\* denotes protein degradation bands. (E–H) Gene expression analysis (qRT-PCR) of the indicated genes in WT and *set4Δ* strains treated with DMSO or 56  $\mu\text{g/ml}$  ketoconazole for 6 hr. The indicated mRNA transcript levels were normalized to *ACT1* and set relative to the DMSO-treated WT [indicated as (-) Keto]. Error bars represent the SD of three biological replicates each with three technical repeats. Gene expression and Western blot analyses were performed using the BY4741 strain.

the ketoconazole-treated samples were set relative to their respective gene in DMSO-treated conditions and normalized to *ACT1*.

To characterize the induction of *SET4* in response to ketoconazole, we treated WT cells with 56  $\mu\text{g/ml}$  ketoconazole and cells were collected over time. As determined by qRT-PCR analysis, *SET4* transcript levels peaked at 6 hr (20-fold increase) postketoconazole treatment (Figure 2C). *ACT1* was used to normalize *SET4* mRNA levels and the ketoconazole-treated samples were set relative to the DMSO WT cells, indicated by the zero time point. To determine if the increase in *SET4* expression following ketoconazole treatment correlated with an increase in protein levels, *Set4* and *Set3* were N-terminally 3XFLAG tagged, using our N-ICE plasmid tagging

system, at their respective loci and expressed from their endogenous promoter (Zhang *et al.* 2016). Western blot analysis of the 3XFLAG-*Set4* strain showed an increase in *Set4* protein following 3 and 6 hr of ketoconazole treatment (Figure 2D, lanes 6 and 5, respectively). Surprisingly, *Set4* levels were undetectable in the DMSO-treated sample, indicating that *Set4* expression is extremely low or not expressed under standard yeast growth conditions (Figure 2D, lane 7). Western blot analysis of 3XFLAG-*Set3* protein levels showed minimal differences in *Set3* protein levels in the DMSO- and ketoconazole-treated samples (Figure 2D, lanes 1 and 2, respectively). G6PDH was used as a loading control. The untagged WT was used as a negative control for ketoconazole- and DMSO-treated strains (Figure 2D, lanes 3 and 4, respectively). Overall, these



**Figure 3** Hypoxia induces Set4 expression. (A and B) *SET4* transcript level was determined in WT cells grown under aerobic conditions or 8 hr of hypoxia by qRT-PCR analysis. \*\*\*\*  $P < 0.0001$ . (B) Expression of *SET1-SET6* was determined in WT cells grown under aerobic conditions or 8 hr of hypoxia by qRT-PCR analysis. Gene expression analyses were set relative to the aerobic WT using the 18S rRNA (*RDN18-1*) as the internal control to normalize transcript levels. Data were analyzed from three biological replicates with three technical replicates. Error bars represent SD. *SET4* was the only gene that significantly changed in expression (\*\*\*\*  $P < 0.0001$ ). (C) Western blot analysis of Set4 protein induction over time under hypoxia. Aerobic (A) and hypoxia (H). The untagged WT was used as a negative control. (D) Western blot analysis of Set4 protein levels following release from hypoxic conditions. Lane 1 represents Set4 protein levels following 8 hr of hypoxia. Lanes 2–7 indicate Set4 protein levels following release from hypoxic conditions. G6PDH was used as a loading control. Gene expression and Western blot analyses were performed in BY4741 strains.

results demonstrate that *Set4* expression is induced under azole treatment and provides the first evidence of an inducible SET domain-containing protein in *S. cerevisiae* and, to our knowledge, in other eukaryotes.

#### ***Set4* does not affect the expression of genes known to be involved in azole resistance**

In recent years, fungal pathogens such as *Candida albicans* and *C. glabrata* have developed resistance to azole antifungal drugs by upregulating the gene encoding the azole drug target, *ERG11*, or upregulating genes encoding the ABC transporters *PDR5* and *PDR11* (Cowen *et al.* 2014; Whaley *et al.* 2016; Campoy and Adrio 2017). To determine if the azole resistance observed in a *set4Δ* strain is correlated with an upregulation of *ERG11*, *PDR5*, or *PDR11* compared to WT; qRT-PCR analysis was performed at the indicated genes in WT and *set4Δ* strain treated with DMSO or 56 μg/ml ketoconazole for 6 hr. However, no statistical difference in the expression of *ERG11*, *ERG3*, *PDR5*, and *PDR11* between WT and the *set4Δ* strain was observed (Figure 2, E–H). Therefore, *Set4* does not mediate drug resistance through known resistant genes but may regulate other genes that are unknown to be involved in azole drug resistance.

#### ***Set4* expression is highly induced under hypoxic conditions**

Because yeast are not typically exposed to azole drugs, we wanted to determine a physiological condition where *Set4* is induced. Interestingly, azole drugs decrease cellular ergosterol levels through inhibition of *Erg11* (Odds *et al.* 2003; Campoy and Adrio 2017). A similar condition occurs when yeast are grown under low oxygen such as hypoxia or anaerobic conditions (Rosenfeld and Beauvoit 2003; Espenshade

and Hughes 2007; Ishtar Snoek and Yde Steensma 2007). Under hypoxia, *ERG* genes are repressed in strains that have full-length *HAP1*, and many of the enzymes in the ergosterol biosynthesis pathway have limited function since they are oxygen dependent (Rosenfeld and Beauvoit 2003; Hon *et al.* 2005; Hickman and Winston 2007). To determine if *Set4* expression increased under hypoxia, we performed qRT-PCR analysis on WT cells grown under aerobic conditions or 8 hr of hypoxia. Strikingly, *SET4* transcript levels were highly upregulated (~200-fold) under hypoxia (Figure 3A). *SET4* transcript levels were normalized to *RDN18-1* and set relative to the aerobic WT strain. These results demonstrate that hypoxia is a physiological condition that significantly induces *SET4* expression in *S. cerevisiae*.

To determine if genes encoding other SET domain proteins modulate their expression in response to hypoxia, we analyzed *SET1-SET6* transcript levels by qRT-PCR under aerobic and hypoxic conditions (Figure 3B). *SET4* transcript levels increased significantly ( $P < 0.0001$ ) under hypoxic conditions (~200-fold), whereas no significant gene expression changes were observed for the remaining genes encoding SET domain proteins (Figure 3B). *RDN18-1* levels were used to normalize transcript levels and each gene was set to the appropriate aerobic sample. These results are consistent with the *SET4* induction under ketoconazole, indicating that conditions that decrease cellular ergosterol levels lead to induction of *Set4* expression.

To determine if the *Set4* protein was also induced under hypoxic conditions, we grew the 3×FLAG-*Set4* strain driven from the endogenous *SET4* promoter over time. Western blot analysis of the 3×FLAG-*Set4* strain showed an increase in *Set4* protein levels after 3 hr of hypoxia (Figure 3C, lane 2). *Set4* proteins levels peaked at 6 hr and remained detectable

through 12 hr of hypoxic treatment (Figure 3C, lanes 3–5). To further characterize *Set4* protein expression levels, we examined how rapidly *Set4* expression decreased when switching from hypoxic to aerobic conditions. The 3×FLAG-*Set4* strain was grown under hypoxic conditions for 8 hr and released into aerobic shaking conditions (Figure 3D). Cells were collected 5, 10, 15, 30, and 60 min following the switch from hypoxia to aerobic conditions (Figure 3D). Western blot analysis of 3×FLAG-*Set4* protein levels demonstrated that *Set4* expression decreased rapidly and is undetectable following 60 min of aerobic growth (Figure 3D, lanes 2–6). G6PDH was used as a loading control and the untagged WT strains grown under aerobic or hypoxic conditions were used as negative controls (Figure 3C, lanes 6 and 7, and Figure 3D, lane 7). These results show that *Set4* expression is tightly regulated under aerobic conditions. Altogether, our results suggest that proper *Set4* expression is needed under environmental conditions that alter sterol levels so that appropriate cellular adaptation occurs.

### ***Set4* is necessary for global gene expression changes under hypoxic conditions**

Transcriptional changes play a major role in yeast adaptation to various environmental conditions, and therefore vast epigenetic changes must occur for yeast to adapt and survive under hypoxic conditions (Ter Linde *et al.* 1999; Hickman *et al.* 2011; Bendjilali *et al.* 2017). To determine if induction of *Set4* modulates hypoxic gene expression and adaptation in response to hypoxia, we performed RNA-sequencing analysis on the BY4741 isogenic WT and *set4Δ* strains grown under hypoxic conditions for 8 hr. After filtering the data, 5900 expressed genes were identified in the analysis covering >90% of the *S. cerevisiae* genome. Principal component analysis (PCA) was performed between aerobic and hypoxic samples. PCA showed that the biological replicates of each strain and condition cluster together (Figure 4A). Additionally, PCA demonstrated that the WT hypoxia, WT aerobic, and *set4Δ* hypoxia clusters are distinct from one another (Figure 4A). Importantly, the *set4Δ* and WT hypoxic sample sets are distinctly different but cluster closer together than the WT aerobic sample set (Figure 4A). This difference in sample clustering suggests that global changes in gene expression occur under hypoxia and when *SET4* is deleted (Figure 4A).

EdgeR analysis was used to identify the differentially expressed genes (DEGs) under hypoxia vs. aerobic conditions in WT samples. A total of 2346 significantly upregulated genes (40% of the genes analyzed) and 2314 significantly downregulated genes (39% of the genes analyzed) were identified (Figure 4B and File S1). PCA and DEG analysis demonstrated by the volcano scatterplot  $\{-\log_2$  [false discovery rate (FDR)],  $y$ -axis $\}$  vs. the fold change ( $x$ -axis) of the DEGs indicate that the hypoxic WT strain is substantially and statistically different from the aerobic WT strain (Figure 4, A and B). Functional analysis indicated that a large number of genes induced under hypoxia are involved in cell stress, carbohydrate metabolism, cell wall maintenance, and lipid metabolism

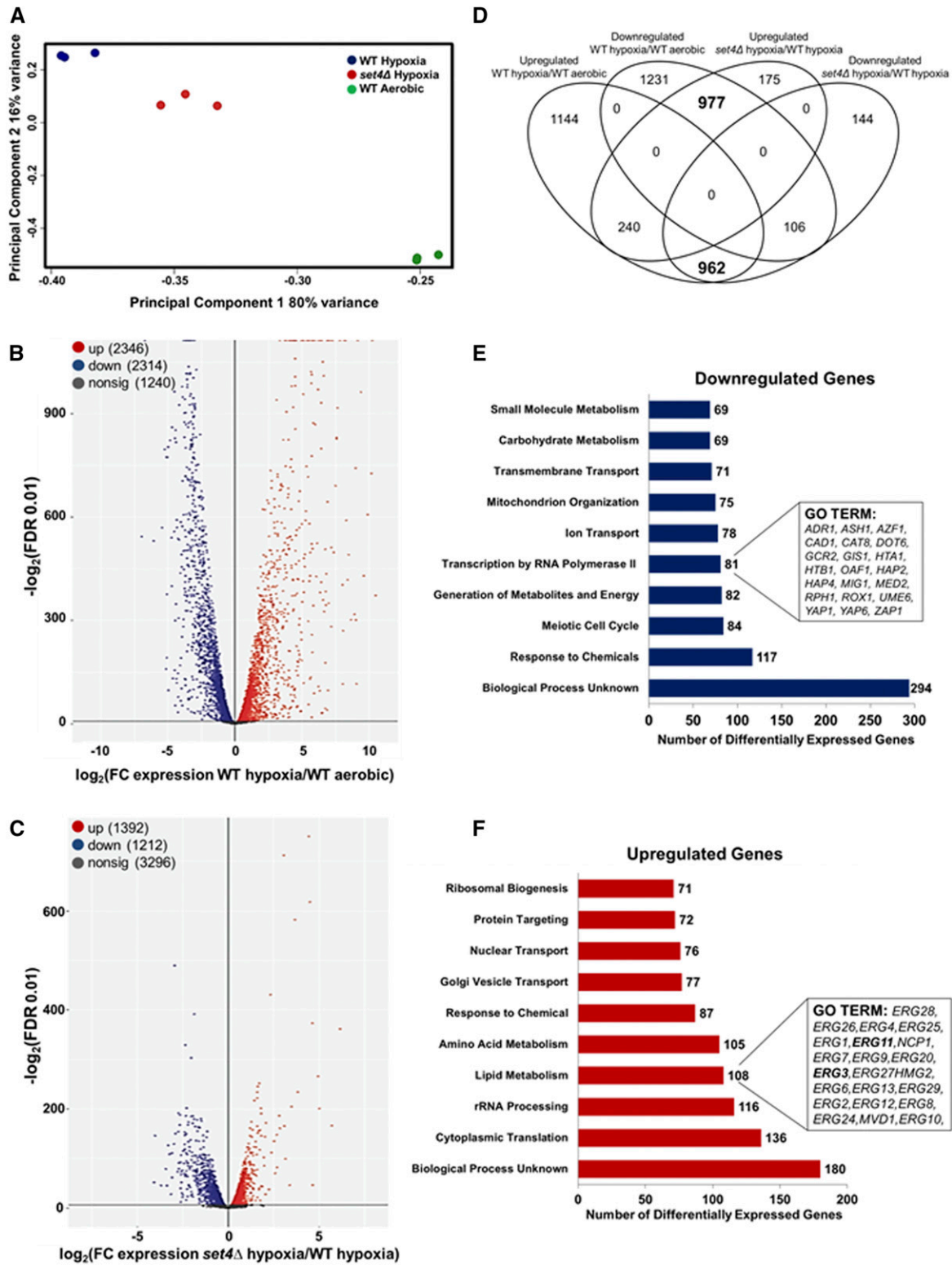
(File S1). Many genes identified in our genome-wide analysis have previously been found to respond to hypoxic conditions (Hickman *et al.* 2011; Bendjilali *et al.* 2017). For example, several studies—including this one—have found the following genes upregulated under hypoxia: *ANB1*, *HEM13*, *CYC7*, and *COX5b*; whereas *CYC1* and *COX5a* were downregulated under hypoxia (File S1) (Ter Linde *et al.* 1999; Hickman and Winston 2007; Bendjilali *et al.* 2017). Furthermore, many cell wall genes are upregulated, including the *DAN/TIR* mannoprotein genes and the seripauperin (*PAU*) genes (File S1) (Ter Linde *et al.* 1999; Rachidi *et al.* 2000; Abramova *et al.* 2001; Cohen *et al.* 2001; Hickman *et al.* 2011; Bendjilali *et al.* 2017). In addition, the sterol transport genes *PDR11* and *AUS1* were upregulated (File S1) (Wilcox *et al.* 2002; Kohut *et al.* 2011).

The PCA and volcano plots also showed a statistically significant difference between the hypoxic *set4Δ* strain and the hypoxic WT (Figure 4, A and C). EdgeR analysis determined that 1392 genes were significantly upregulated (23% of genes analyzed) and 1212 genes were significantly downregulated (20% of genes analyzed) (FDR < 0.01) (Figure 4C and File S2). To determine the number of genes that overlap between the different strains and conditions tested, we plotted a Venn diagram representing the upregulated and downregulated genes for each comparison group (Figure 4D). According to the Venn diagram, a high degree of overlap was observed between genes that exhibited increased expression in WT cells under hypoxia and genes that were significantly downregulated in the *set4Δ* strain under hypoxia (962 genes,  $P < 10^{-52}$  by Fisher's exact test) (Figure 4D). In contrast, the intersection between the DEGs exhibiting decreased expression in WT and a *set4Δ* strain was not larger than predicted by chance alone (106 genes,  $P > 0.99$ ). These results reveal that *Set4* is required directly or indirectly for the activation of a significant number of genes under hypoxia (Figure 4D).

In addition, 977 genes were downregulated in the comparison between WT under hypoxia and aerobic conditions but upregulated in the *set4Δ* strain under hypoxia, suggesting that *Set4* is required to directly or indirectly repress these genes (Figure 4D). This high degree of overlap was also statistically significant and indicates that *Set4* is required for the repression of a significant number of genes under hypoxia (977 genes,  $P < 10^{-52}$  by Fisher's exact test). The intersection between the DEGs exhibiting increased expression in both WT and a *set4Δ* strain was not larger than predicted by chance alone (106 genes,  $P > 0.99$ ) (Figure 4D). Together, these comparisons imply that *Set4* is necessary to promote the transcriptional switch from aerobic to hypoxic conditions. Lastly, the GoSLIM database on the *Saccharomyces* Genome Database was used to categorize the biological processes, molecular functions, and pathways of the DEGs (Figure 4, E and F, and File S3). The top-10 gene ontology (GO) terms are shown in Figure 4, E and F. Additional GO terms are shown in File S3.

Many of the DEGs that were downregulated in the *set4Δ* strain included genes encoding meiotic cell cycle factors, factors that respond to chemical stress, genes involved in metabolite





**Figure 4** Set4 alters global levels of gene expression under hypoxic conditions. The genome-wide changes in gene expression under hypoxia were performed using BY4741 WT and *set4Δ* strains. (A) The PCA for WT and *set4Δ* hypoxic samples relative to WT aerobic samples based on the counts per million. (B) Volcano plot showing the significance [ $-\log_2(\text{FDR})$ , y-axis] vs. the fold change (x-axis) of the DEGs identified in the WT hypoxic samples relative to WT aerobic samples. (C) Volcano plot showing the significance [ $-\log_2(\text{FDR})$ , y-axis] vs. the fold change (x-axis) of the DEGs identified in the *set4Δ* hypoxic samples relative to WT hypoxic samples. Genes with significant differential expression ( $\text{FDR} < 0.01$ ) in (B and C) are highlighted in red or blue for up- and downregulated genes, respectively. Gray highlighted genes are considered nonsignificant. (D) Venn diagram showing the number of genes identified as differentially expressed ( $\text{FDR} < 0.01$ ). Bold numbers indicate a high overlap of genes predicted to be in common by chance based on

generation, and factors that mediate transcription by RNA polymerase II (Figure 4E and File S3). A majority of the DEGs that were upregulated in the *set4Δ* strain included genes involved in cytoplasmic translation, rRNA processing, amino acid metabolism, and lipid metabolism; suggesting that these genes are dependent on *Set4* for repression under hypoxia (Figure 4F and File S3). Many genes upregulated in a *set4Δ* strain were not categorized in any biological process (File S3). Of these uncharacterized genes, 18 are *PAU* genes which have been hypothesized to play a role in cell wall remodeling (File S3) (Luo and van Vuuren 2009). Interestingly, 19 out of 20 genes involved in ergosterol metabolism were significantly upregulated in the *set4Δ* strain compared to WT (Figure 4F). These results indicate that *Set4* is required for repressing genes involved in ergosterol metabolism. Because *ERG* genes are repressed under hypoxic conditions, it is likely that *Set4* induction facilitates repression of the *ERG* genes and the entire ergosterol biosynthesis pathway for proper sterol homeostasis, cell wall remodeling, and uptake of exogenous sterols.

#### ***Set4* directly targets and enriches at ergosterol gene promoters under hypoxia**

Cellular ergosterol levels are known to decrease in WT cells under hypoxia (Hickman *et al.* 2011; Bendjilali *et al.* 2017). To verify that *ERG* gene expression decreases in response to hypoxia, we performed qRT-PCR analysis in FY2609 WT cells grown under aerobic conditions and 3, 6, and 9 hr of hypoxia. *ERG11* and *ERG3* transcript levels decreased significantly in WT cells following 6 hr (65 and 81%, respectively) and 9 hr of hypoxic treatment (85 and 93%, respectively) (Figure 5, A and B). The significant decrease in *ERG11* and *ERG3* transcript levels correlates with the time at which *Set4* expression peaked under hypoxic conditions (Figure 3C). In addition, our RNA-sequencing data clearly indicate that one role for *Set4* is to repress genes involved in ergosterol biosynthesis (Figure 4F). To validate our RNA-sequencing result, we analyzed the *ERG11* and *ERG3* transcript levels by qRT-PCR in the FY2609 WT and *set4Δ* strains grown under hypoxia for 3, 6, and 9 hr of hypoxia. Consistent with our RNA-sequencing analysis, a *set4Δ* strain had approximately three- to sixfold more expression of *ERG11* and *ERG3* mRNA transcript levels compared to the WT strain at the 6 and 9 hr time points (Figure 5, C and D). To determine if this increase in *ERG* gene expression under hypoxia was specific to the *set4Δ* strain, we analyzed the *ERG11* and *ERG3* mRNA levels in WT, *set4Δ*, *set3Δ*, and the double deletion *set4Δset3Δ* strain under hypoxic conditions. In contrast to aerobic conditions, *ERG11* and *ERG3* transcript levels are not altered in a *set3Δ* strain under hypoxic conditions (Figure S2 in File S5). Furthermore,

*ERG11* and *ERG3* transcript levels significantly increased in the *set4Δ* and *set4Δset3Δ* strains, demonstrating that *Set4* specifically mediates *ERG* gene repression under hypoxic conditions (Figure S2 in File S5).

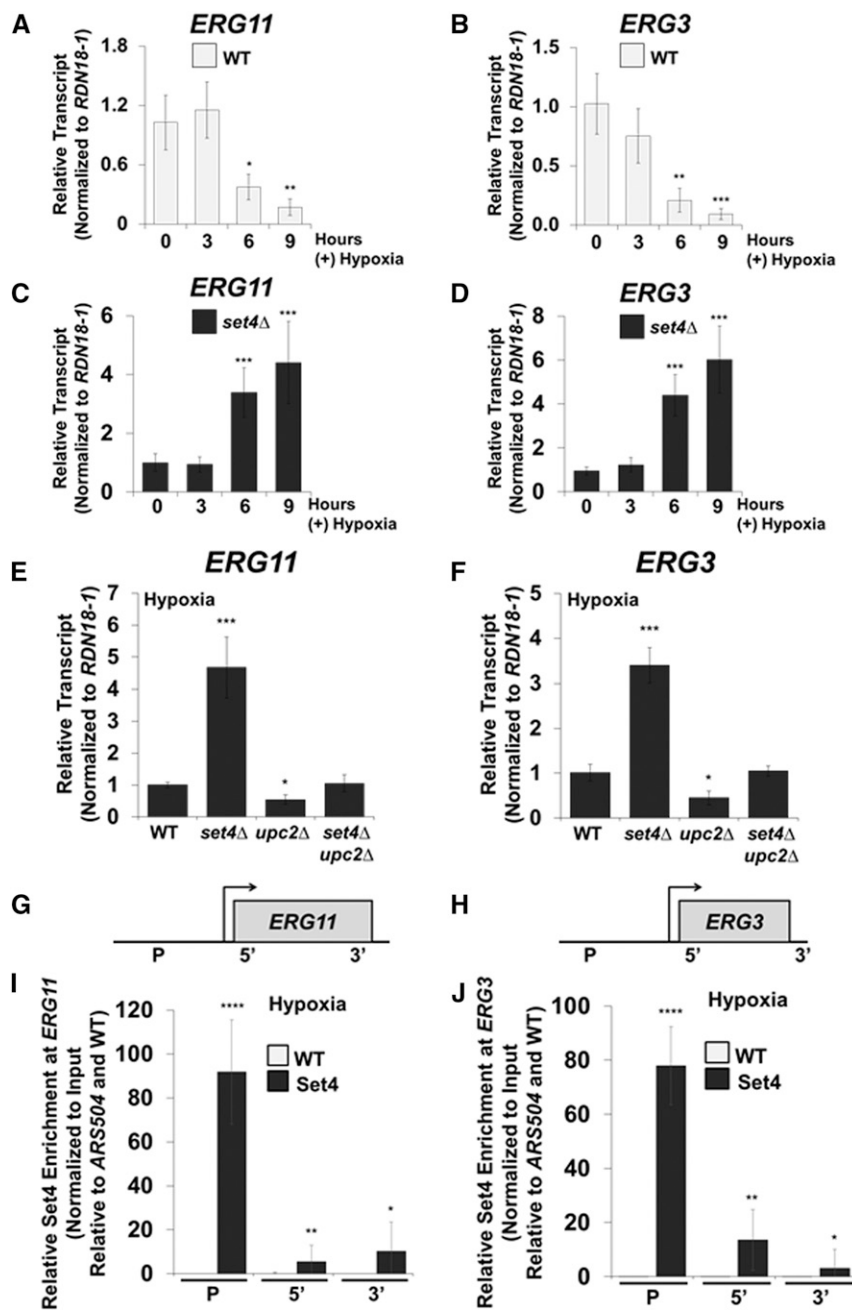
Both *ERG11* and *ERG3* mRNA transcript levels increase significantly in a *set4Δ* strain under hypoxia, suggesting that a transcriptional activator upregulated these *ERG* genes in the absence of *Set4*. Previously it has been shown that the transcriptional activator, *Upc2*, targets *ERG* genes under sterol limiting conditions (Vik and Rine 2001; Davies *et al.* 2005; Davies and Rine 2006). To determine whether *ERG11* and *ERG3* gene expression is upregulated by *Upc2* when *SET4* is deleted, qRT-PCR analysis was performed under hypoxic conditions in the WT, *set4Δ*, *upc2Δ*, and *set4Δupc2Δ* strains. Interestingly, both *ERG11* and *ERG3* mRNA transcript levels failed to increase in the *upc2Δ* and *set4Δupc2Δ* strains under hypoxic conditions, indicating that *Upc2* is required to upregulate *ERG11* and *ERG3* expression in the absence of *Set4* (Figure 5, E and F). Additionally, *ERG11* and *ERG3* transcript levels significantly decreased in a *upc2Δ* strain but not in the *set4Δupc2Δ* strain, suggesting that *Set4* represses these genes further when *UPC2* is deleted (Figure 5, E and F). The mRNA transcript levels were normalized to *RDN18-1* and set relative to the hypoxic WT strain. Together, these data imply that *Set4* and *Upc2* oppose one another to regulate *ERG* gene expression under hypoxic conditions.

Because *Set4* and *Upc2* both regulate the expression of *ERG11* and *ERG3* (Figure 5, E and F), we hypothesized that *Set4* directly targets *ERG* gene promoters to facilitate gene repression under hypoxia. To determine *Set4* binding at *ERG* promoters, we performed ChIP analysis using our published ZipChIP method (Harmeyer *et al.* 2015). Probe sets were designed to the promoter and the 5' and 3' ORFs of *ERG11* and *ERG3* (Figure 5, G and H). Interestingly, ZipChIP analysis showed that 3×FLAG-*Set4* was significantly enriched at the promoter regions of the *ERG11* and *ERG3* loci when compared with the untagged WT (Figure 5, I and J). In contrast, 3×FLAG-*Set4* was minimally detected at the 5' ORF and the 3' ORF (Figure 5, I and J). The qRT-PCR analysis was normalized to DNA input levels and set relative to the autonomously replicating sequence, *ARS504*, and WT untagged strain. Altogether, our data demonstrate that *Set4* represses ergosterol genes and directly targets the promoters of *ERG11* and *ERG3*.

Although our data show that *Set4* directly represses *ERG* genes, RNA sequencing suggests that *Set4* may be needed for the direct activation of select downregulated DEGs, including oxidative stress (*CTT1*) and sporulation (*SPS100*) genes (Figure 4E). To test whether *Set4* directly targets these genes, our

---

Fisher's exact test ( $P < 10^{-52}$ ). (E and F) GO terms of the *Set4*-dependent DEGs under hypoxic conditions. Downregulated genes refer to the DEGs that are dependent on *Set4* for activation and the upregulated genes refer to the DEGs that are dependent on *Set4* for repression. Significantly enriched groups of GO terms were identified for the DEGs from *set4Δ* and WT hypoxic samples. Bar plots show the number of DEGs in each GO group that are dependent on *Set4* under hypoxia. The number of genes in each GO group is shown to the right of each bar. Genes identified are shown in the inset boxes.



**Figure 5** Set4 represses ergosterol genes and directly targets the promoters of *ERG11* and *ERG3* under hypoxia. (A and B) The mRNA transcript levels of *ERG11* and *ERG3* were determined in WT cells grown under aerobic or hypoxic conditions over time. qRT-PCR expression analysis in the WT strain was set relative to the aerobic or hypoxic WT using the 18S ribosome rRNA (*RDN18-1*) as the internal control to normalize transcript levels. (C and D) qRT-PCR analysis of *ERG11* and *ERG3* expression in *set4Δ* cells grown under aerobic or hypoxic conditions for 3, 6, or 9 hr. *ERG11* and *ERG3* expression in the *set4Δ* strain were normalized to *RDN18-1* and set to the WT strain at the same relative time point. Data were analyzed from three biological replicates that had three technical replicates each. Error bars represent SD. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$ . (E and F) Gene expression analysis (qRT-PCR) of *ERG11* and *ERG3* under hypoxic conditions in WT, *set4Δ*, *upc2Δ*, and *set4Δupc2Δ*. The mRNA transcript levels were normalized to *RDN18-1* and set relative to WT grown under hypoxia. \*  $P < 0.05$ , \*\*\*  $P < 0.005$ . (G and H) Schematics of *ERG11* and *ERG3* loci with the specified positions of ChIP probes. (I and J) ChIP analysis of Set4 enriched at *ERG11* and *ERG3* was performed under hypoxic conditions using antibodies specific to a 3×FLAG tag for the detection of Set4. ChIP analyses were normalized to DNA input samples and set relative to the *ARS504* and untagged WT. Error bars represent SD for three biological replicates with three technical replicates each. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*\*  $P < 0.0001$ . Gene expression and ChIP analyses were performed using the FY2609 strain.

ZipChIP analysis was performed. ChIP analysis showed minor Set4 enrichment at the promoters of *CTT1* and *SPS100* (5- and 17-fold, respectively) (Figure S3 in File S5). In addition, Set4 was slightly enriched (22-fold) at a constitutively expressed gene, *PMA1*, which was not differentially expressed between WT and *set4Δ* strains (Figure S3 in File S5). In contrast, Set4 promoter enrichment at *ERG11* (92-fold) and *ERG3* (79-fold) was significantly higher than at *CTT1*, *SPS100*, or *PMA1* (compare Figure 5, I and J, with Figure S3 in File S5). These results demonstrate that Set4 has low promoter enrichment at DEGs found to be downregulated in a *set4Δ* strain. We expect that Set4 is either non-specifically binding at these promoters or plays an indirect

role in the activation of these genes. Additional studies are needed to better understand how Set4 functions at downregulated DEGs.

#### **Set4 is targeted to ergosterol gene promoters by Hap1 under hypoxic conditions**

Our data show that Set4 functions as a direct repressor of *ERG* genes under hypoxic conditions (Figure 4 and Figure 5). Because Set4 enriches at the promoter of *ERG* genes, we hypothesized that Set4 may associate with a transcription factor to help facilitate gene repression under hypoxia. To determine the Set4-interacting partners under hypoxic conditions, we performed an immunoprecipitation assay with untagged

WT and 3×FLAG-Set4 strains using an anti-FLAG-specific antibody from whole cell lysate harvested from hypoxic yeast cells. The immunoprecipitated samples were separated by SDS-PAGE and trypsin digested. The resulting peptides from the 3×FLAG-Set4 and untagged WT immunoprecipitation hypoxic samples were eluted and analyzed by MS. Peptide hits revealed that Set4 co-immunoprecipitated with the transcription factor Hap1 and the transcriptional corepressor Tup1 under hypoxic conditions, suggesting that Set4 functions in a complex with Hap1 and Tup1 (File S4). Interestingly, Hap1 and Tup1 have been shown to repress ERG genes under hypoxic conditions (Hickman and Winston 2007).

To confirm that Hap1 and Tup1 repress ERG genes under our hypoxic conditions, we analyzed transcript levels by qRT-PCR in *hap1Δ* and *tup1Δ* strains, normalized to *RDN18-1* and set relative to the WT strain. Consistent with published data, *ERG11* and *ERG3* expression levels increased significantly in the *hap1Δ* and *tup1Δ* strains (Figure 6, A and B) (Davies and Rine 2006; Hickman and Winston 2007). To determine if Hap1 directly targets the *ERG11* and *ERG3* loci under hypoxia, our ZipChIP was performed (Harmeyer *et al.* 2015). ChIP analysis showed that Myc-Hap1 is significantly enriched at the promoters of *ERG11* and *ERG3* (Figure 6, E and F), consistent with previous studies (Davies and Rine 2006; Hickman and Winston 2007). Importantly, the promoter probe sets were designed near (within one nucleosome distance) the Hap1 binding sites identified in the promoters of *ERG11* (−788 and −641) and *ERG3* (−358) by the YEASTRACT repository (Figure 6, C and D).

To determine if Hap1 is necessary for Set4 recruitment to ERG gene promoters, we analyzed 3×FLAG-Set4 enrichment in WT and *hap1Δ* strains. Strikingly, 3×FLAG-Set4 promoter localization was significantly decreased in the *hap1Δ* strain, demonstrating that Hap1 is necessary for Set4 recruitment to ERG gene promoters under hypoxia (Figure 6, G and H). No significant difference in Set4 enrichment between the WT and *hap1Δ* strains was observed at the 5′ regions (Figure 6, G and H). Additionally, Western blot analysis demonstrated that 3×FLAG-Set4 protein expression levels were unaffected in a *hap1Δ* strain under hypoxia, verifying that the loss of Set4 enrichment at the promoter in a *hap1Δ* strain was not due to changes in global Set4 expression levels (Figure S4 in File S5). Altogether, these results demonstrate that Set4 associates and functions with Hap1 and the corepressor, Tup1, to maintain ergosterol homeostasis during hypoxic conditions.

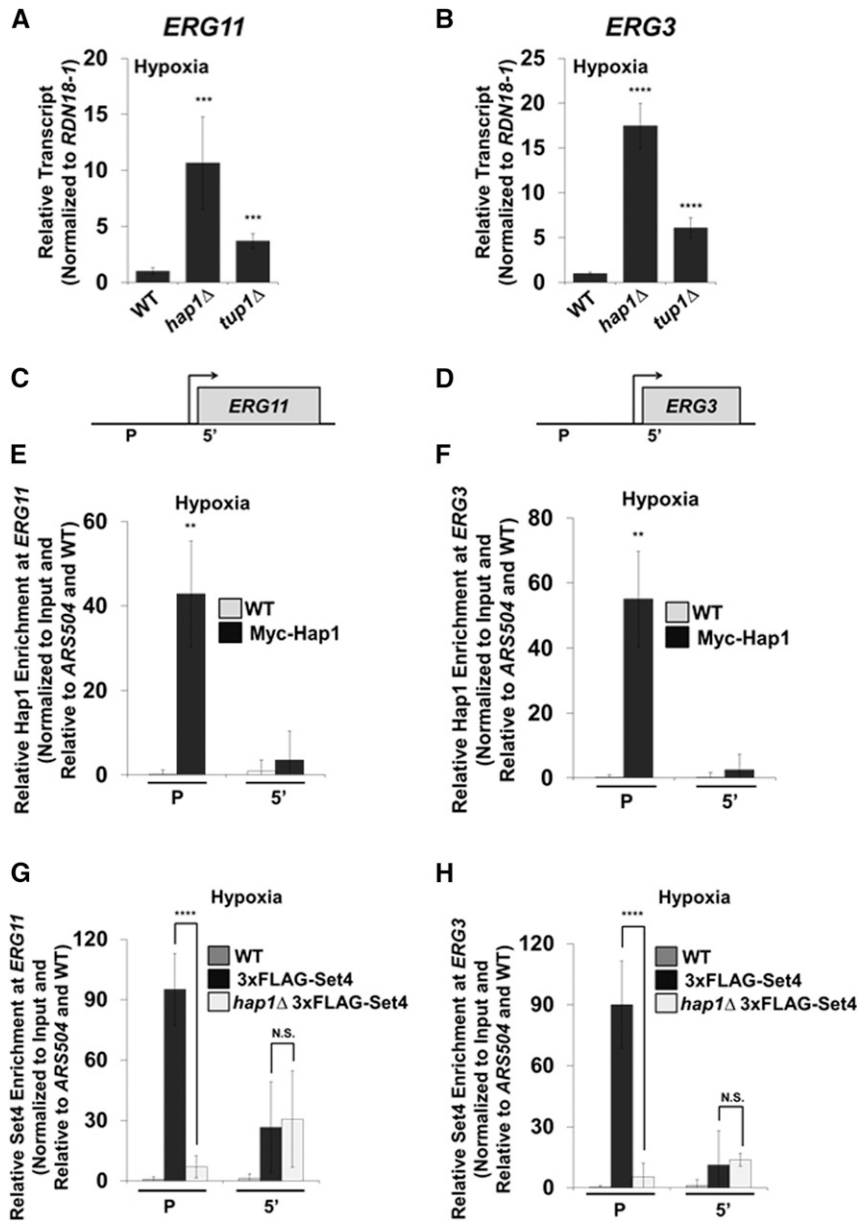
#### **Upc2 and Ecm22 are necessary for Set4 expression under hypoxic conditions and azole antifungal drug treatment**

Because Set4 is induced under hypoxia and azole treatment, we wanted to determine the transcription factor(s) responsible for inducing Set4 expression. Previously, it has been shown that the transcriptional activators, Upc2 and Ecm22, upregulate gene targets in response to sterol depletion (Vik and Rine 2001; Davies *et al.* 2005; Davies and Rine 2006; Hickman *et al.* 2011). Under sterol depletion, Upc2 has been

suggested to be the primary transcriptional regulator of ergosterol biosynthesis genes (Vik and Rine 2001; Davies *et al.* 2005; Davies and Rine 2006). Because sterol depletion occurs under hypoxia, we hypothesized that Upc2 and Ecm22, a paralog of Upc2, were necessary for Set4 induction under hypoxic conditions. First, the *SET4* promoter was analyzed for Upc2 and Ecm22 binding sites (Figure 7A). Upc2 and Ecm22 are sterol regulatory element binding proteins that have been suggested to recognize the 7-bp DNA sequence TCGTATA known as the sterol regulatory element; Upc2 also recognizes the sequence TCGTTAAA (Vik and Rine 2001). The *SET4* promoter contains three potential Upc2/Ecm22 binding sites predicted by the YEASTRACT repository database, including two binding sites for Upc2 and Ecm22 (−1384 and −633), and one site for Upc2 (−1025) (Figure 7A). To determine if *SET4* expression is dependent on Upc2 and Ecm22 under hypoxia, we generated the following single and double deletion strains—*upc2Δ*, *ecm22Δ*, and *upc2Δecm22Δ*—and analyzed each deletion strain for *SET4* mRNA levels. As predicted, the *upc2Δ* strain resulted in a significant decrease (72%) of *SET4* transcript levels under hypoxia; however, the *ecm22Δ* strain did not affect *SET4* expression (Figure 7B). Nevertheless, the *upc2Δecm22Δ* double deletion strain abolished *SET4* transcript levels under hypoxia (Figure 7B).

To determine if the decrease in *SET4* transcript levels in the *upc2Δ* and *upc2Δecm22Δ* strains correlate with a decrease in protein levels, we generated the corresponding deletions—*upc2Δ*, *ecm22Δ*, and *upc2Δecm22Δ*—in the 3×FLAG-Set4 strain. Western blot analysis of the 3×FLAG-Set4 strains showed a moderate reduction in Set4 protein levels in the *upc2Δ* strain and a slight decrease in the *ecm22Δ* strain under hypoxia (Figure 7C, lanes 4 and 6). However, Set4 protein levels were completely abolished in the *upc2Δecm22Δ* double deletion strain (Figure 7C, lane 8), confirming that Upc2 and Ecm22 are both necessary for the hypoxic-mediated induction of Set4 (Figure 7C). G6PDH was used as a loading control and an untagged WT grown under hypoxia was used as a negative control.

Studies have shown that Upc2 upregulates several gene targets including ERG genes, DAN/TIR genes, and PAU genes under antifungal drug treatment (Vik and Rine 2001; Wilcox *et al.* 2002; Davies *et al.* 2005; Davies and Rine 2006; Gallo-Ebert *et al.* 2013; Woods and Höfken 2016). To determine if Set4 expression is also dependent on Upc2 under azole drug treatment, we determined *SET4* expression levels following 6 hr of treatment with 56 μg/ml ketoconazole in the WT, *upc2Δ*, *ecm22Δ*, and *upc2Δecm22Δ* strains. Consistent with hypoxic conditions, *SET4* transcript levels significantly decreased in the *upc2Δ* strain under ketoconazole treatment, albeit lower than observed under hypoxic conditions (Figure 7, B and D). Additionally, *SET4* transcript levels were unchanged in the *ecm22Δ* strain (Figure 7D), while the *upc2Δecm22Δ* strains show similar *SET4* transcript levels as the *upc2Δ* (Figure 7D); suggesting that *SET4* induction is completely dependent on Upc2. To determine if Set4 protein levels are solely dependent on Upc2 under azole drug treatment, we



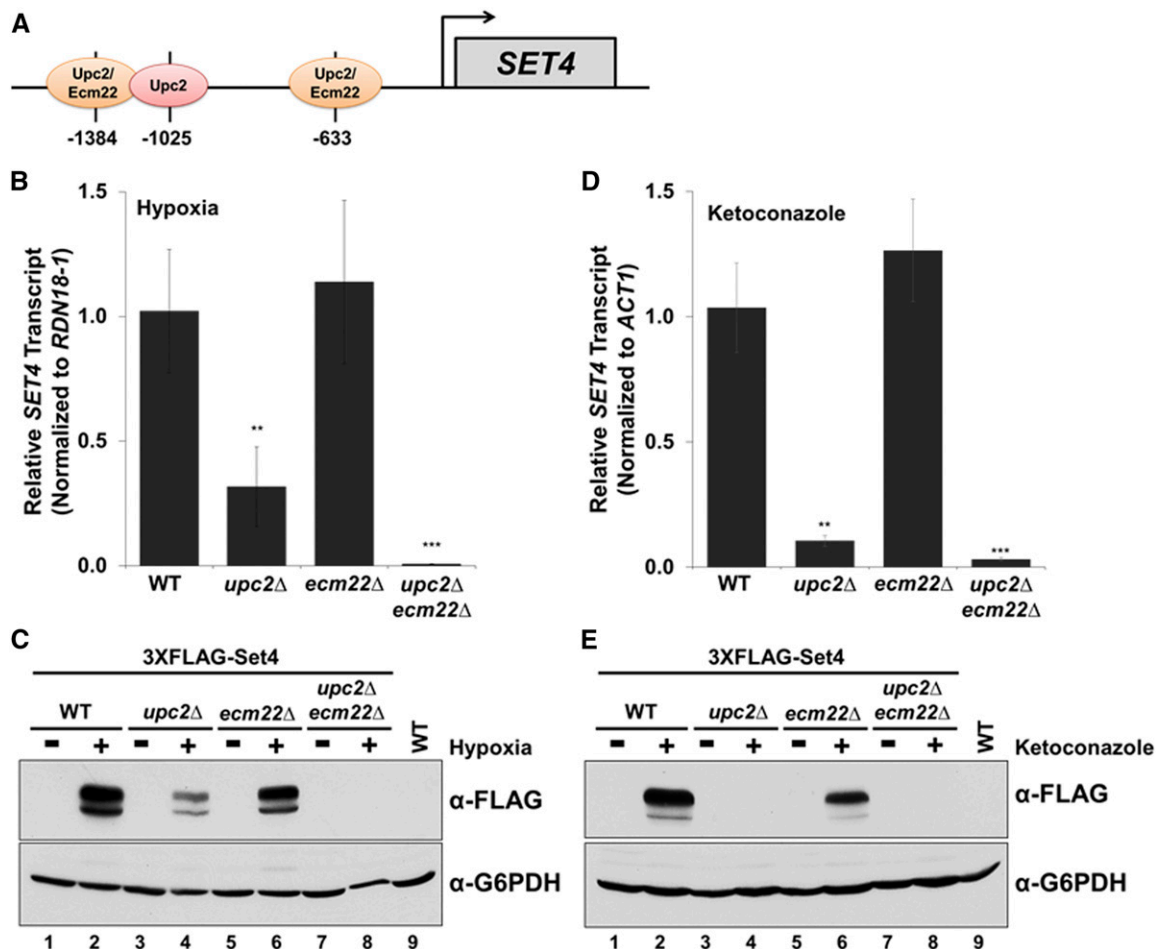
**Figure 6** The transcriptional repressor, Hap1, is required for Set4 binding to the promoters of ergosterol genes under hypoxic conditions. (A and B) Relative transcript levels of *ERG11* and *ERG3* were determined following 8 hr of hypoxia in WT, *hap1Δ*, and *tup1Δ* strains using qRT-PCR analysis. Transcript levels were set relative to the WT strain and expression levels were normalized to *RDN18-1*. \*\*\*  $P < 0.005$ , \*\*\*\*  $P < 0.0001$ . (C and D) Schematics of *ERG11* and *ERG3* loci with the specified positions of ChIP probes. (E and F) ChIP analysis of Hap1 under hypoxia at *ERG11* and *ERG3*. \*\*  $P < 0.005$ . (G and H) ChIP analysis of Set4 under hypoxia in WT and *hap1Δ* strains at *ERG11* and *ERG3*. ChIP analyses were normalized to DNA input samples and set relative to the untagged WT and the *ARS504* loci. Error bars represent SD for three biological replicates with three technical replicates each. Gene expression and ChIP analyses were performed using the FY2609 strain. \*\*\*\*  $P < 0.0001$ . N.S., no significant difference.

performed Western blot analysis in the 3×FLAG-Set4 strains containing *upc2Δ*, *ecm22Δ*, and *upc2Δecm22Δ* double deletions. Consistent with *SET4* transcript levels, Set4 protein expression is undetectable in the *upc2Δ* and *upc2Δecm22Δ* double deletion strains treated with ketoconazole (Figure 7E, lanes 4 and 8). These results indicate that Upc2 is the primary transcription factor required to induce Set4 expression, while Ecm22 is partially responsible for Set4 expression in the absence of Upc2 under hypoxic conditions. In contrast, Upc2 is exclusively responsible for upregulating Set4 levels under azole treatment.

#### An *erg3Δ* strain induces the expression of Set4 under untreated aerobic conditions

Because hypoxia and azole drug treatment are conditions that decrease ergosterol and increase sterol precursors, we

hypothesized that Upc2 would upregulate Set4 expression under aerobic conditions in strains that are deficient in producing ergosterol but still produce precursor sterols. To test this hypothesis, we deleted the genes encoding the enzymes (*ERG6*, *ERG2*, *ERG3*, *ERG5*, and *ERG4*) needed for the final five steps of the ergosterol biosynthesis pathway (Figure 8A), and *SET4* expression levels were examined by qRT-PCR. Intriguingly, the *erg3Δ* strain resulted in a significant increase (~10-fold) in *SET4* transcript levels, whereas *SET4* expression increased threefold or less in the remaining ergosterol mutants (Figure 8B). To determine if Set4 protein levels induce in an *erg3Δ* strain, we deleted *ERG3* in the 3×FLAG-Set4 strain. Western blot analysis revealed that 3×FLAG-Set4 protein was expressed in the *erg3Δ* strain, suggesting that increased levels of an ergosterol precursor(s) in the *erg3Δ* strain are involved in upregulating Set4 expression (Figure



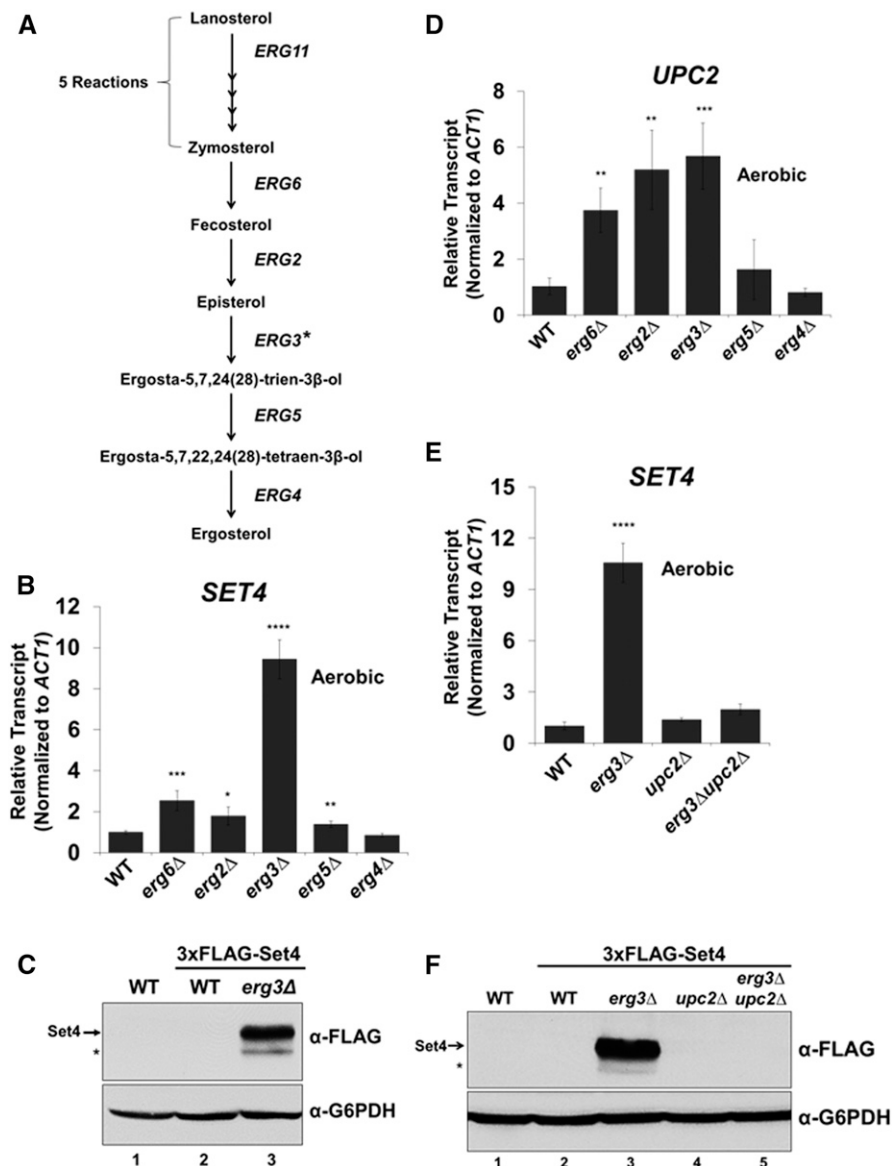
**Figure 7** Set4 expression under hypoxia or azole drug-treated conditions is primarily dependent on Upc2. (A) Schematic of the Upc2 and Ecm22 binding sites located in the *SET4* promoter. (B and D) Using the indicated strains, *SET4* expression was determined by qRT-PCR analysis. *SET4* expression levels were normalized to *RDN18-1* (hypoxia) or *ACT1* (ketoconazole) and set relative to the aerobic or DMSO-treated WT strain. Data were analyzed from three biological replicates with three technical repeats. Each error bar represents SD. \*\*  $P < 0.01$  and \*\*\*  $P < 0.005$ . (C and E) Western blot analysis of Set4 levels under the indicated conditions. G6PDH was used as a loading control. Gene expression and Western blot analyses were performed using the BY4741 strain.

8C and Figure S5 in File S5). Ergosterol precursors generated in an *erg3* $\Delta$  strain are episterol, ergosta-7-enol, ergosta-7,22,24(28)-trienol, and ergosta-7,22-dienol (Figure S5 in File S5) (Bard *et al.* 1993; Sanglard *et al.* 2003; Martel *et al.* 2010; Vale-Silva *et al.* 2012). Because *Set4* expression is dependent on *Upc2* under sterol depletion (Figure 7), *UPC2* transcript levels were analyzed in the ergosterol mutants to determine whether *UPC2* levels were upregulated in response to decreased ergosterol levels. The qRT-PCR analysis showed that *UPC2* expression is significantly upregulated in the *erg6* $\Delta$ , *erg2* $\Delta$ , and *erg3* $\Delta$  strains and not significantly affected in the *erg5* $\Delta$  and *erg4* $\Delta$  strains; indicating that a decrease in ergosterol does not result in *Upc2* induction (Figure 8D). To test if *Upc2* is required for *Set4* expression in the *erg3* $\Delta$  strain, we generated the *erg3* $\Delta$ *upc2* $\Delta$  double deletion strain. Consistent with the requirement of *Upc2* for the induction of *Set4* under azole and hypoxic conditions, *SET4* transcript level failed to increase in the *erg3* $\Delta$ *upc2* $\Delta$  strain (Figure 8E). In addition, Western blot analysis revealed that 3 $\times$ FLAG-*Set4* expression was

undetectable in the *erg3* $\Delta$ *upc2* $\Delta$  double deletion strain, verifying that *Set4* expression is solely dependent on *Upc2* in the *erg3* $\Delta$  strain (Figure 8F, lane 5). Altogether, our data indicate that accumulation of a sterol precursor(s) in the *erg3* $\Delta$  strain works together with *Upc2* to induce *Set4* expression.

## Discussion

In this study, we established that *Set4* is an inducible SET domain-containing protein that governs azole antifungal drug resistance, and directly represses ergosterol genes under hypoxic conditions by a Hap1-dependent mechanism. Initially, we identified that *Set1*, *Set3*, and *Set4* govern drug efficacy to the medically relevant class of azole antifungal drugs. Upon further investigation, we surprisingly discovered that *Set4* is not constitutively expressed under standard yeast growth conditions but is induced by the sterol-responsive transcriptional activators, *Upc2* and *Ecm22*, under hypoxic

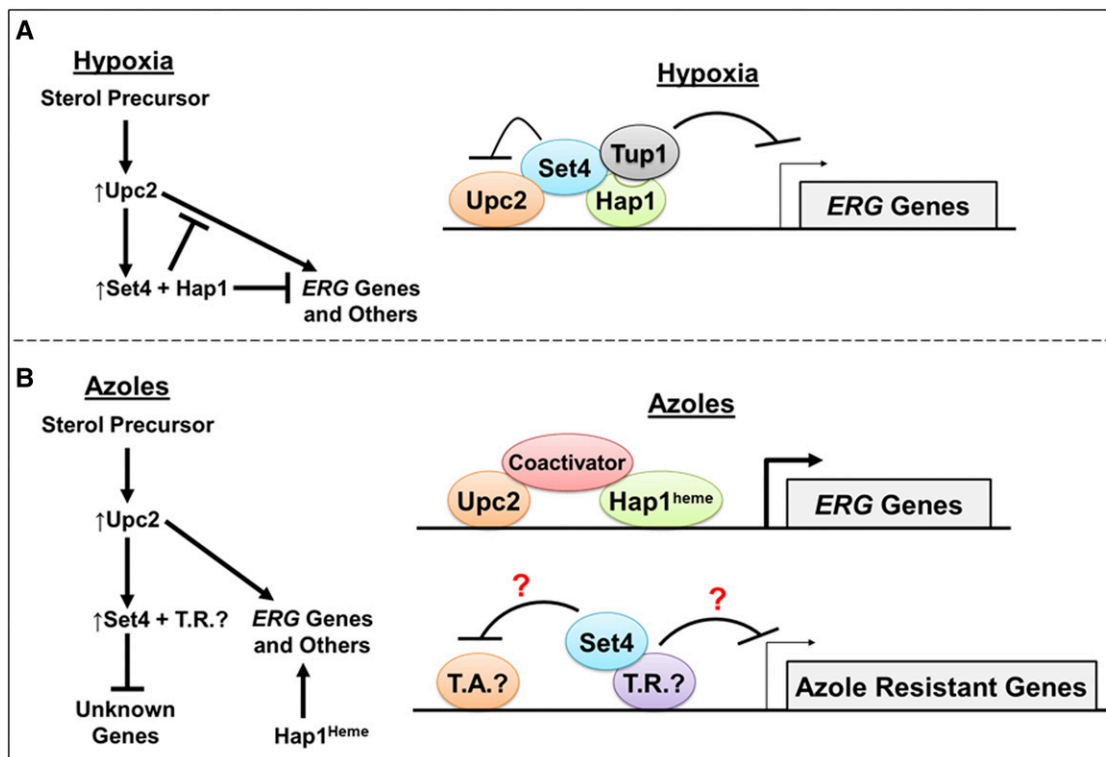


**Figure 8** Set4 is constitutively expressed in an *erg3* $\Delta$  strain under aerobic conditions by a precursor sterol and Upc2. (A) Modified pathway showing the final steps in ergosterol biosynthesis. The \* on *ERG3* represents potential pathways that modify episterol to generate additional sterol precursors (see Figure S5 in File S5). (B) qRT-PCR analysis of *SET4* transcript levels in ergosterol mutant strains under aerobic conditions. Transcript levels were normalized to *ACT1* and set relative to WT. (C) Western blot analysis of 3 $\times$ FLAG-Set4 levels in WT and *erg3* $\Delta$  strain under aerobic conditions. The \* indicates a likely protein degradation band. (D) Gene expression analysis by qRT-PCR of *UPC2* in indicated strains under aerobic conditions. (E) *SET4* mRNA levels determined by qRT-PCR in WT, *erg3* $\Delta$ , *upc2* $\Delta$ , and *erg3* $\Delta$ *upc2* $\Delta$  under aerobic conditions. (B, D, E) \*  $P < 0.05$ , \*\*  $P < 0.005$ , \*\*\*  $P < 0.0005$ , \*\*\*\*  $P < 0.0001$ . (F) Western blot analysis of Set4 protein levels in strains from E under aerobic conditions. G6PDH was used as a loading control and an untagged WT was used as a negative control. The band under the Set4 band likely indicates protein degradation and is indicated by \*. Gene expression data were normalized to *ACT1* and set relative to WT. Error bars represent SD of three biological replicates. Gene expression and Western blot analyses were performed using the BY4741 strain.

conditions or azole drug treatment. Furthermore, we determined that *Set4* modulates global gene expression under ergosterol-limiting conditions, and that under hypoxia *Set4* is recruited to promoters by *Hap1* to directly repress *ERG3* and *ERG11* expression. Therefore, *Set4* plays a key role in cellular adaptation to environmental conditions that deplete ergosterol levels. Interestingly, *Set4* expression is also upregulated in an untreated *erg3* $\Delta$  strain but not in an *erg3* $\Delta$ *upc2* $\Delta$  double deletion strain, suggesting *Upc2* and a precursor sterol(s)—but not ergosterol—control *Set4* levels. Altogether, we have identified a new sterol-signaling pathway involving precursor sterols, sterol-sensing transcription factors, and *Set4*, which govern azole antifungal drug resistance and regulates the repression of sterol genes under hypoxic conditions.

*Set4* has previously been defined as the *Set3* paralog because *SET4* was likely derived from *SET3* during the whole-genome duplication of the *Saccharomycotina* species, and has been identified in *S. cerevisiae*, *S. bayanus*, *S. castellii*, and the

opportunistic fungal pathogen *C. glabrata* (Pijnappel *et al.* 2001; Byrne and Wolfe 2005). Both *Set4* and *Set3* contain an N-terminal PHD finger and C-terminal SET domain; however, neither *Set3* nor *Set4* have been identified to have histone methyltransferase activity (Pijnappel *et al.* 2001). Additionally, the *Set4* homologs UpSET (*Drosophila melanogaster*) and MLL5 (human, long isoform) have not been shown to have histone methyltransferase activity (Pijnappel *et al.* 2001; Fujiki *et al.* 2009; Sebastian *et al.* 2009; Mas *et al.* 2016; Zhang *et al.* 2017). On the other hand, MLL5 (human, short isoform) has been reported to have H3K4 methylation activity after MLL5 (short) has been post-translationally modified by O-GlcNac transferase (Fujiki *et al.* 2009). However, in *S. cerevisiae*, histone methylation by *Set4* was undetectable both *in vitro* and *in vivo* when testing global levels of H3K4 methylation under aerobic and hypoxic conditions by Western blot analysis (Figure S6 in File S5). Currently, it is unknown whether *Set4* has methyltransferase activity on histones or nonhistone substrates.



**Figure 9** Sterol precursor(s) lead to Upc2-facilitated induction of Set4 to repress genes required for antifungal drug resistance and sterol homeostasis under hypoxic conditions. (A) Model under hypoxia: Sterol-facilitated induction of Set4 by Upc2 mediates repression of *ERG* genes by association with the transcriptional repressors Hap1 and Tup1 to block the function of the transcriptional activator, Upc2. Hap1 under hypoxic conditions is heme independent and is considered a transcriptional repressor. Activation of *ERG* genes by Upc2 is blocked by the presence of Set4 and Hap1 as indicated by the inhibitory symbol. S288C strains expressing the *hap1-Ty1* gene fusion partially repress *ERG* gene expression; whereas yeast strains expressing *HAP1* will fully repress *ERG* genes. (B) Model under azole drug treatment: Sterol-facilitated induction of Set4 by Upc2 mediates repression of genes involved in azole resistance and likely associates with a transcriptional repressor (T.R.) to block a transcriptional activator (T.A.). Under azole treatment, *ERG* genes are Set4 independent but are activated by Upc2, Hap1<sup>heme</sup>, and coactivators. Hap1 under aerobic conditions is bound to heme and is considered a transcriptional activator.

Like *Set3*, *Set4* alters global gene expression; however, the mechanism by which *Set4* modulates ergosterol gene expression is unknown but is likely different from *Set3* (Kim *et al.* 2012). For example, loss of *Set4* increases *ERG11* and *ERG3* transcript levels, whereas a deletion of *Set3* does not affect these genes under hypoxia (Figure S2 in File S5). Under aerobic conditions, the *Set3* complex has been shown to enrich at the 5' regions of gene targets through associations with H3K4me2 and the Ser5-phosphorylated C-terminal domain of RNA polymerase II (Kim and Buratowski 2009; Govind *et al.* 2010). In addition, unlike the PHD finger of *Set3*, the PHD finger of *Set4* does not bind to H3K4 methylated histones (Shi *et al.* 2007). Together, this suggests that *Set3* and *Set4* have evolved distinct biochemical functions.

Overall, our studies support a model where Upc2 induces *SET4* to block Upc2-mediated *ERG* gene activation by a repressive complex consisting of *Set4*, *Hap1*, and *Tup1* under hypoxia (Figure 5, E and F, and Figure 9A). Therefore, the *Set4-Hap1-Tup1* complex functions in a feedback mechanism to regulate Upc2-mediated *ERG* gene activation (Figure 9A). In support of this hypothesis, deletion of *SET4* results in

increased expression of *ERG11* and *ERG3* compared to WT, whereas this increase is abolished in the *upc2Δ* and *set4Δupc2Δ* strains under hypoxia (Figure 5, E and F).

Importantly, *ERG11* and *ERG3* gene expression increased similarly in the BY4741 *set4Δ* strain compared to the FY2609 *set4Δ* strain, indicating that neither the BY4741 strain expressing the *hap1-Ty1* gene fusion nor the FY2609 strain expressing the WT *HAP1* affects the function of *Set4* (Figure S7 in File S5). Interestingly, *ERG11* and *ERG3* transcript levels increase by about twofold in the BY4741 WT strain; whereas *ERG* gene expression significantly decreases (Figure 5) in the FY2609 strain when switched to hypoxic conditions (Figure S8 in File S5). To our knowledge, this is the first observation indicating that the BY4741 strain expressing the *hap1-Ty1* gene fusion is partially defective in repressing *ERG* genes (Figure S8 in File S5 and File S1). However, this is consistent with the findings that strains expressing the *hap1-Ty1* gene fusion are partially defective in Hap1-mediated gene activation under aerobic conditions (Gaisne *et al.* 1999; Tamura *et al.* 2004).

In contrast, *ERG11*, *ERG3*, *PDR5*, and *PDR11* expression increased similarly in WT and *set4Δ* strains treated with



ketoconazole, indicating that these are *Set4*-independent genes (Figure 2, E–H). Therefore, under azole treatment, *Set4* must regulate the expression of alternative gene targets unknown to play a role in azole drug resistance. We speculate that *Set4* does not associate with *Hap1* since *Hap1* is bound to heme under aerobic conditions and acts as a transcriptional activator for *ERG* genes (Figure 9B). Genome-wide gene expression analysis and/or genetic suppressor screens will identify *Set4* gene targets that contribute to azole drug resistance.

An important result revealed from our study was the observation that *Set4* expression levels are upregulated in an *erg3Δ* strain (Figure 8). These data suggest that a precursor sterol, not ergosterol, is signaling induction of *Set4* by an *Upc2*-mediated mechanism. In an *erg3Δ* strain, several ergosterol precursors have been indicated to accumulate under aerobic, untreated conditions in yeast such as episterol, ergosta-7-enol, ergosta-7,22,24(28)-trienol and ergosta-7,22-dienol (Figure S5 in File S5) (Sanglard *et al.* 2003; Martel *et al.* 2010; Vale-Silva *et al.* 2012). In a recent study, *Upc2* was shown to bind ergosterol sequestering *Upc2* outside of the nucleus; however, when ergosterol is depleted using treatment with fluconazole, *Upc2* localizes to the nucleus (Yang *et al.* 2015). In an *erg3Δ* strain, ergosterol cannot be synthesized; therefore, the sterol precursors that accumulate in *erg3Δ* strain might bind to *Upc2* or another factor, leading to *Upc2* nuclear localization and *Set4* expression. Understanding how *Set4* functions in eukaryotic sterol homeostasis can provide insight into how SET domain proteins alter gene expression in hypoxic environments such as the human gut microbiota or tumor microenvironment. Furthermore, understanding the role of *Set4* in drug resistance can provide insight into the development of new or improved antifungal drugs as well as develop novel strategies for treating fungal infections and antifungal drug resistance.

## Acknowledgments

We thank Peter Cheung for reviewing this manuscript and Fred Winston for yeast strains. We thank the Purdue Bioinformatics Core for pipelines and bioinformatics software. This work was supported by the Purdue Department of Biochemistry, Bilsland Fellowship (to N.D.S.); the Purdue Biochemistry Bird Stair Fellowship (to N.D.S.); Ross Fellowship (to K.M.B.); Purdue Center for Cancer Research (grant P30CA023168: DNA Sequencing Shared Resource and Collaborative Core for Cancer Bioinformatics at Purdue); the Walther Cancer Foundation; and the Indiana University Simon Cancer Center (grant P30CA082709). Additional funding support was provided by the Purdue Executive Vice President for Research and Partnerships award (to S.D.B.), Purdue Center for Cancer Research Challenge Award (to S.D.B.), National Institute of Food and Agriculture 1007570 (S.D.B.), and the National Institutes of Health R01 EY-024905 (V.M.W.).

## Literature Cited

- Abramova, N., O. Sertil, S. Mehta, and C. V. Lowry, 2001 Reciprocal regulation of anaerobic and aerobic cell wall mannoprotein gene expression in *Saccharomyces cerevisiae*. *J. Bacteriol.* 183: 2881–2887.
- Agarwal, A. K., P. D. Rogers, S. R. Baerson, M. R. Jacob, K. S. Barker *et al.*, 2003 Genome-wide expression profiling of the response to polyene, pyrimidine, azole, and echinocandin antifungal agents in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 278: 34998–35015.
- Alimardani, P., M. Regnacq, C. Moreau-Vauzelle, T. Ferreira, T. Rossignol *et al.*, 2004 SUT1-promoted sterol uptake involves the ABC transporter Aus1 and the mannoprotein Dan1 whose synergistic action is sufficient for this process. *Biochem. J.* 381: 195–202.
- Bard, M., N. D. Lees, T. Turi, D. Craft, L. Cofrin *et al.*, 1993 Sterol synthesis and viability of *erg11* (cytochrome P450 lanosterol demethylase) mutations in *Saccharomyces cerevisiae* and *Candida albicans*. *Lipids* 28: 963–967.
- Becerra, M., L. J. Lombardia-Ferreira, N. C. Hauser, J. D. Hoheisel, B. Tizon *et al.*, 2002 The yeast transcriptome in aerobic and hypoxic conditions: effects of *hap1*, *rox1*, *rox3* and *srb10* deletions. *Mol. Microbiol.* 43: 545–555.
- Bendjilali, N., S. MacLeon, G. Kalra, S. D. Willis, A. K. Hossian *et al.*, 2017 Time-course analysis of gene expression during the *Saccharomyces cerevisiae* hypoxic response. *G3 (Bethesda)* 7: 221–231.
- Briggs, S. D., M. Bryk, B. D. Strahl, W. L. Cheung, J. K. Davie *et al.*, 2001 Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. *Genes Dev.* 15: 3286–3295.
- Byrne, K. P., and K. H. Wolfe, 2005 The yeast gene order browser: combining curated homology and syntenic context reveals gene fate in polyploid species. *Genome Res.* 15: 1456–1461.
- Campoy, S., and J. L. Adrio, 2017 Antifungals. *Biochem. Pharmacol.* 133: 86–96.
- Cohen, B. D., O. Sertil, N. E. Abramova, K. J. Davies, and C. V. Lowry, 2001 Induction and repression of *DAN1* and the family of anaerobic mannoprotein genes in *Saccharomyces cerevisiae* occurs through a complex array of regulatory sites. *Nucleic Acids Res.* 29: 799–808.
- Cowen, L. E., D. Sanglard, S. J. Howard, P. D. Rogers, and D. S. Perlin, 2014 Mechanisms of antifungal drug resistance. *Cold Spring Harb. Perspect. Med.* 5: a019752.
- Davies, B. S., and J. Rine, 2006 A role for sterol levels in oxygen sensing in *Saccharomyces cerevisiae*. *Genetics* 174: 191–201.
- Davies, B. S., H. S. Wang, and J. Rine, 2005 Dual activators of the sterol biosynthetic pathway of *Saccharomyces cerevisiae*: similar activation/regulatory domains but different response mechanisms. *Mol. Cell. Biol.* 25: 7375–7385.
- Dupont, S., G. Lemetais, T. Ferreira, P. Cayot, P. Gervais *et al.*, 2012 Ergosterol biosynthesis: a fungal pathway for life on land? *Evolution* 66: 2961–2968.
- Espenshade, P. J., and A. L. Hughes, 2007 Regulation of sterol synthesis in eukaryotes. *Annu. Rev. Genet.* 41: 401–427.
- Fingerman, I. M., C. L. Wu, B. D. Wilson, and S. D. Briggs, 2005 Global loss of Set1-mediated H3 Lys4 trimethylation is associated with silencing defects in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 280: 28761–28765.
- Flowers, S. A., K. S. Barker, E. L. Berkow, G. Toner, S. G. Chadwick *et al.*, 2012 Gain-of-function mutations in *UPC2* are a frequent cause of *ERG11* upregulation in azole-resistant clinical isolates of *Candida albicans*. *Eukaryot. Cell* 11: 1289–1299.
- Fujiki, R., T. Chikanishi, W. Hashiba, H. Ito, I. Takada *et al.*, 2009 GlcNAcylation of a histone methyltransferase in retinoic-acid-induced granulopoiesis. *Nature* 459: 455–459.

- Fytlovich, S., M. Gervais, C. Agrimonti, and B. Guiard, 1993 Evidence for an interaction between the CYP1(HAP1) activator and a cellular factor during heme-dependent transcriptional regulation in the yeast *Saccharomyces cerevisiae*. *EMBO J.* 12: 1209–1218.
- Gaisne, M., A. M. Becam, J. Verdier, and C. J. Herbert, 1999 A 'natural' mutation in *Saccharomyces cerevisiae* strains derived from S288c affects the complex regulatory gene HAP1 (CYP1). *Curr. Genet.* 36: 195–200.
- Gallo-Ebert, C., M. Donigan, H. Y. Liu, F. Pascual, M. Manners *et al.*, 2013 The yeast anaerobic response element AR1b regulates aerobic antifungal drug-dependent sterol gene expression. *J. Biol. Chem.* 288: 35466–35477.
- Gatta, A. T., L. H. Wong, Y. Y. Sere, D. M. Calderon-Norena, S. Cockcroft *et al.*, 2015 A new family of StART domain proteins at membrane contact sites has a role in ER-PM sterol transport. *Elife* 4: e07253.
- Govind, C. K., H. Qiu, D. S. Ginsburg, C. Ruan, K. Hofmeyer *et al.*, 2010 Phosphorylated Pol II CTD recruits multiple HDACs, including Rpd3C(S), for methylation-dependent deacetylation of ORF nucleosomes. *Mol. Cell* 39: 234–246.
- Harmeyer, K. M., P. F. South, B. Bishop, J. Ogas, and S. D. Briggs, 2015 Immediate chromatin immunoprecipitation and on-bead quantitative PCR analysis: a versatile and rapid ChIP procedure. *Nucleic Acids Res.* 43: e38.
- Hickman, M. J., and F. Winston, 2007 Heme levels switch the function of Hap1 of *Saccharomyces cerevisiae* between transcriptional activator and transcriptional repressor. *Mol. Cell. Biol.* 27: 7414–7424.
- Hickman, M. J., D. Spatt, and F. Winston, 2011 The Hog1 mitogen-activated protein kinase mediates a hypoxic response in *Saccharomyces cerevisiae*. *Genetics* 188: 325–338.
- Hon, T., H. C. Lee, Z. Hu, V. R. Iyer, and L. Zhang, 2005 The heme activator protein Hap1 represses transcription by a heme-independent mechanism in *Saccharomyces cerevisiae*. *Genetics* 169: 1343–1352.
- Ishtar Snoek, I. S., and H. Yde Steensma, 2007 Factors involved in anaerobic growth of *Saccharomyces cerevisiae*. *Yeast* 24: 1–10.
- Joshua, I. M., and T. Höfken, 2017 From lipid homeostasis to differentiation: old and new functions of the zinc cluster proteins Ecm22, Upc2, Sut1 and Sut2. *Int. J. Mol. Sci.* 18: E772.
- Kathiravan, M. K., A. B. Salake, A. S. Chothe, P. B. Dudhe, R. P. Watode *et al.*, 2012 The biology and chemistry of antifungal agents: a review. *Bioorg. Med. Chem.* 20: 5678–5698.
- Kim, T., and S. Buratowski, 2009 Dimethylation of H3K4 by Set1 recruits the Set3 histone deacetylase complex to 5' transcribed regions. *Cell* 137: 259–272.
- Kim, T., Z. Xu, S. Clauder-Munster, L. M. Steinmetz, and S. Buratowski, 2012 Set3 HDAC mediates effects of overlapping noncoding transcription on gene induction kinetics. *Cell* 150: 1158–1169.
- Kohut, P., D. Wustner, L. Hronska, K. Kuchler, I. Hapala *et al.*, 2011 The role of ABC proteins Aus1p and Pdr11p in the uptake of external sterols in yeast: dehydroergosterol fluorescence study. *Biochem. Biophys. Res. Commun.* 404: 233–238.
- Kwast, K. E., P. V. Burke, and R. O. Poyton, 1998 Oxygen sensing and the transcriptional regulation of oxygen-responsive genes in yeast. *J. Exp. Biol.* 201: 1177–1195.
- Kwast, K. E., L. C. Lai, N. Menda, D. T. James, III, S. Aref *et al.*, 2002 Genomic analyses of anaerobically induced genes in *Saccharomyces cerevisiae*: functional roles of Rox1 and other factors in mediating the anoxic response. *J. Bacteriol.* 184: 250–265.
- Lombardia, L. J., J. L. Cadahia-Rodriguez, M. A. Freire-Picos, M. I. Gonzalez-Siso, A. M. Rodriguez-Torres *et al.*, 2000 Transcript analysis of 203 novel genes from *Saccharomyces cerevisiae* in *hap1* and *rox1* mutant backgrounds. *Genome* 43: 881–886.
- Luo, Z., and H. J. van Vuuren, 2009 Functional analyses of PAU genes in *Saccharomyces cerevisiae*. *Microbiology* 155: 4036–4049.
- Markovich, S., A. Yekutieli, I. Shalit, Y. Shadkchan, and N. Osherov, 2004 Genomic approach to identification of mutations affecting caspofungin susceptibility in *Saccharomyces cerevisiae*. *Antimicrob. Agents Chemother.* 48: 3871–3876.
- Martel, C. M., J. E. Parker, O. Bader, M. Weig, U. Gross *et al.*, 2010 Identification and characterization of four azole-resistant *erg3* mutants of *Candida albicans*. *Antimicrob. Agents Chemother.* 54: 4527–4533.
- Mas, Y. M. S., M. Barbon, C. Teyssier, H. Demene, J. E. Carvalho *et al.*, 2016 The human mixed lineage leukemia 5 (MLL5), a sequentially and structurally divergent SET domain-containing protein with no intrinsic catalytic activity. *PLoS One* 11: e0165139.
- Mersman, D. P., H. N. Du, I. M. Fingerman, P. F. South, and S. D. Briggs, 2012 Charge-based interaction conserved within histone H3 lysine 4 (H3K4) methyltransferase complexes is needed for protein stability, histone methylation, and gene expression. *J. Biol. Chem.* 287: 2652–2665.
- Mouritsen, O. G., and M. J. Zuckermann, 2004 What's so special about cholesterol? *Lipids* 39: 1101–1113.
- Muren, E., M. Oyen, G. Barmark, and H. Ronne, 2001 Identification of yeast deletion strains that are hypersensitive to brefeldin A or monensin, two drugs that affect intracellular transport. *Yeast* 18: 163–172.
- Odds, F. C., A. J. Brown, and N. A. Gow, 2003 Antifungal agents: mechanisms of action. *Trends Microbiol.* 11: 272–279.
- Parks, L. W., and W. M. Casey, 1995 Physiological implications of sterol biosynthesis in yeast. *Annu. Rev. Microbiol.* 49: 95–116.
- Pijnappel, W. W. M. P., D. Schaft, A. Roguev, A. Shevchenko, H. Tekotte *et al.*, 2001 The *S. cerevisiae* SET3 complex includes two histone deacetylases, Hos2 and Hst1, and is a meiotic-specific repressor of the sporulation gene program. *Genes Dev.* 15: 2991–3004.
- Rachidi, N., M. J. Martinez, P. Barre, and B. Blondin, 2000 *Saccharomyces cerevisiae* PAU genes are induced by anaerobiosis. *Mol. Microbiol.* 35: 1421–1430.
- Rosenfeld, E., and B. Beauvoit, 2003 Role of the non-respiratory pathways in the utilization of molecular oxygen by *Saccharomyces cerevisiae*. *Yeast* 20: 1115–1144.
- Sanglard, D., F. Ischer, T. Parkinson, D. Falconer, and J. Bille, 2003 *Candida albicans* mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. *Antimicrob. Agents Chemother.* 47: 2404–2412.
- Sebastian, S., P. Sreenivas, R. Sambasivan, S. Cheedipudi, P. Kandalla *et al.*, 2009 MLL5, a trithorax homolog, indirectly regulates H3K4 methylation, represses cyclin A2 expression, and promotes myogenic differentiation. *Proc. Natl. Acad. Sci. USA* 106: 4719–4724.
- Shi, X., I. Kachirskaya, K. L. Walter, J. H. Kuo, A. Lake *et al.*, 2007 Proteome-wide analysis in *Saccharomyces cerevisiae* identifies several PHD fingers as novel direct and selective binding modules of histone H3 methylated at either lysine 4 or lysine 36. *J. Biol. Chem.* 282: 2450–2455.
- South, P. F., I. M. Fingerman, D. P. Mersman, H. N. Du, and S. D. Briggs, 2010 A conserved interaction between the SDI domain of Bre2 and the Dpy-30 domain of Sdc1 is required for histone methylation and gene expression. *J. Biol. Chem.* 285: 595–607.
- South, P. F., K. M. Harmeyer, N. D. Serratore, and S. D. Briggs, 2013 H3K4 methyltransferase Set1 is involved in maintenance of ergosterol homeostasis and resistance to Brefeldin A. *Proc. Natl. Acad. Sci. USA* 110: E1016–E1025.
- Tamura, K., Y. Gu, Q. Wang, T. Yamada, K. Ito *et al.*, 2004 A *hap1* mutation in a laboratory strain of *Saccharomyces cerevisiae* results in decreased expression of ergosterol-related genes and

- cellular ergosterol content compared to sake yeast. *J. Biosci. Bioeng.* 98: 159–166.
- Ter Linde, J. J., and H. Y. Steensma, 2002 A microarray-assisted screen for potential Hap1 and Rox1 target genes in *Saccharomyces cerevisiae*. *Yeast* 19: 825–840.
- Ter Linde, J. J., H. Liang, R. W. Davis, H. Y. Steensma, J. P. van Dijken *et al.*, 1999 Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of *Saccharomyces cerevisiae*. *J. Bacteriol.* 181: 7409–7413.
- Vale-Silva, L. A., A. T. Coste, F. Ischer, J. E. Parker, S. L. Kelly *et al.*, 2012 Azole resistance by loss of function of the sterol  $\Delta^{5,6}$ -desaturase gene (*ERG3*) in *Candida albicans* does not necessarily decrease virulence. *Antimicrob. Agents Chemother.* 56: 1960–1968.
- Vik, A., and J. Rine, 2001 Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 21: 6395–6405.
- Weete, J. D., M. Abril, and M. Blackwell, 2010 Phylogenetic distribution of fungal sterols. *PLoS One* 5: e10899.
- Whaley, S. G., E. L. Berkow, J. M. Rybak, A. T. Nishimoto, K. S. Barker *et al.*, 2016 Azole antifungal resistance in *Candida albicans* and emerging non-*albicans* *Candida* species. *Front. Microbiol.* 7: 2173.
- Wilcox, L. J., D. A. Balderes, B. Wharton, A. H. Tinkelenberg, G. Rao *et al.*, 2002 Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. *J. Biol. Chem.* 277: 32466–32472.
- Woods, K., and T. Höfken, 2016 The zinc cluster proteins Upc2 and Ecm22 promote filamentation in *Saccharomyces cerevisiae* by sterol biosynthesis-dependent and -independent pathways. *Mol. Microbiol.* 99: 512–527.
- Yang, H., J. Tong, C. W. Lee, S. Ha, S. H. Eom *et al.*, 2015 Structural mechanism of ergosterol regulation by fungal sterol transcription factor Upc2. *Nat. Commun.* 6: 6129.
- Zavrel, M., S. J. Hoot, and T. C. White, 2013 Comparison of sterol import under aerobic and anaerobic conditions in three fungal species, *Candida albicans*, *Candida glabrata*, and *Saccharomyces cerevisiae*. *Eukaryot. Cell* 12: 725–738.
- Zhang, L., and A. Hach, 1999 Molecular mechanism of heme signaling in yeast: the transcriptional activator Hap1 serves as the key mediator. *Cell. Mol. Life Sci.* 56: 415–426.
- Zhang, X., W. Novera, Y. Zhang, and L. W. Deng, 2017 MLL5 (KMT2E): structure, function, and clinical relevance. *Cell. Mol. Life Sci.* 74: 2333–2344.
- Zhang, Y., N. D. Serratore, and S. D. Briggs, 2016 N-ICE plasmids for generating N-terminal 3 x FLAG tagged genes that allow inducible, constitutive, or endogenous expression in *Saccharomyces cerevisiae*. *Yeast* 34: 223–235.

Communicating editor: A. Hinnebusch