We determined the changes in transcriptional profiles that occur in the first hour following the transfer of *Candida albicans* to hypoxic growth conditions. The impressive speed of this response is not compatible with current models of fungal adaptation to hypoxia that depend on the depletion of sterol and heme. Functional analysis using Gene Set Enrichment Analysis (GSEA) identified the Sit4 phosphatase, Ccr4 mRNA deacetylase, and Sko1 transcription factor (TF) as potential regulators of the early hypoxic response. Cells mutated in these and other regulators exhibit a delay in their transcriptional responses to hypoxia. Promoter occupancy data for 29 TFs were combined with the transcriptional profiles of 3,111 *in vivo* target genes in a Network Component Analysis (NCA) to produce a model of the dynamic and highly interconnected TF network that controls this process. With data from the TF network obtained from a variety of sources, we generated an edge and node model that was capable of separating many of the hypoxia-upregulated and -downregulated genes. Upregulated genes are centered on Tye7, Upc2, and Mrr1, which are associated with many of the gene promoters that exhibit the strongest activations. The connectivity of the model illustrates the high redundancy of this response system and the challenges that lie in determining the individual contributions of specific TFs. Finally, treating cells with an inhibitor of the oxidative phosphorylation chain mimics most of the early hypoxic profile, which suggests that this response may be initiated by a drop in ATP production.

*Candida albicans* is an opportunistic pathogen responsible for various non-life-threatening infections and is a major cause of morbidity and mortality, particularly in immunosuppressed patients. Its ability to invade multiple body sites and organs requires adaptation to changes in oxygen and carbon dioxide concentrations, temperature, pH, osmolarity, and nutrient availability. Colonization of aerobic body sites, including the skin and mucosal surfaces, as well as of oxygen-poor locations such as internal organs and the gastrointestinal tract, demonstrates that *C. albicans* is adept at acclimating to both normoxia and hypoxia. Under hypoxic conditions, the ability of *C. albicans* to form hyphae, the growth mode dedicated to host invasion and colonization, is enhanced, suggesting a close relationship between the hypoxic response and pathogenesis (1). Furthermore, hypoxia positively affects different biological processes such as chlamydospore formation and the stability of mating-competent opaque cells (1–3). In an earlier study, we demonstrated that *C. albicans* cells growing as biofilms in a flow model present a transcriptional signature similar to that of planktonic cells growing under hypoxic conditions (4). This suggests that the biofilm cells, or at least a subpopulation of the biofilm, are confronting hypoxic conditions.

The majority of studies on sensing, signaling, and transcriptional regulatory mechanisms of hypoxia adaptation in fungi have been conducted on *Saccharomyces cerevisiae*. Homologs of the mammalian SREBP (sterol regulatory element binding protein) transcription factors (TFs) were characterized in several fungi, and their role in the adaptation to low-oxygen environments and low sterol levels was proven (5). The inactive form of SREBP is a membrane-bound protein that requires cleavage by a specific proteolytic machinery in order to be released in the cytosol and then translocate to the nucleus to modulate many hypoxic transcripts, including *ERG* genes. Unfortunately, knowledge gained from budding yeast is not fully applicable to *C. albicans*. Transcriptional regulatory networks and cell signaling pathways often exhibit functional rewiring between different fungal species (6, 7), and the *C. albicans* genome does not contain a functional SREBP homolog. There are also significant metabolic differences; while *S. cerevisiae* ferments sugars to ethanol even under aerobic conditions, *C. albicans* oxidizes carbohydrates through respiration in aerobic conditions and ferments carbohydrates under hypoxic conditions (8).

The transcriptional response to hypoxia has been directly elucidated in some fungal pathogens, including *C. albicans* (9–11) and *Aspergillus fumigatus* (12). Generally, in response to low oxygen concentrations, fungal cells upregulate genes involved in ergosterol (ERG) biosynthesis, fatty acid and iron metabolism, cell wall structure, glycolysis, and fermentation, while respiration genes are repressed (1, 13). The contribution of different TFs to the modulation of specific hypoxic genes has been revealed in different studies. Setiadi et al. (9) have demonstrated the role of the key filamentation regulator Efg1 in the transcriptional activation of unsaturated fatty acid metabolism genes under hypoxic conditions. Additional investigations have established the crucial role of the TF Upc2, the bona fide transcriptional regulator of ERG metabolism in different fungi, in mediating anaerobic growth and
ERG gene inducibility under hypoxic conditions (11, 14, 15). Finally, we and others have shown that Tye7, a primary regulator of the central carbon metabolic circuit, is required for full activation of glycolytic and fermentation genes under hypoxic conditions (10, 16).

While those previous studies on the transcriptional response of \textit{C. albicans} to hypoxia identified similar lists of hypoxia-responsive genes, cells were usually assayed once they were fully adapted to hypoxic conditions (9–11). Since our goal was to better link TF functions to gene expression profiles and to reduce the added complexity of secondary effects, we have instead undertaken a comprehensive genome-wide transcriptional study of \textit{C. albicans} cells experiencing hypoxia over an early time course. The aim of this work was initially to illustrate the different oxygen-controlled biological processes accompanying a quick adaptation to oxygen deprivation. On the basis of earlier studies, we anticipated a complex transcriptional response, and its interpretation was addressed using a variety of improved bioinformatic tools specifically adapted to \textit{C. albicans}. These methods, including Gene Set Enrichment Analysis (GSEA) and Network Component Analysis (NCA) along with node and edge analysis, were extremely useful in deciphering the transcriptional data and to provide biologically and/or mechanistically meaningful insights into the early adaptive hypoxic response. This work illustrates how a systems biology approach can provide a comprehensive view of a complex biological process.

**MATERIALS AND METHODS**

\textit{C. albicans} strains and growth media and conditions. For general propagation and maintenance conditions, cells were cultured at 30°C in yeast extract-peptone-dextrose (YPD) medium supplemented with uridine (2% Bacto peptone, 1% yeast extract, 2% dextrose, and 50 μg/ml uridine). Hypoxic conditions for the expression profiling experiments were as follows: \textit{C. albicans} strain SN148 (arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ/ura3Δ imm434/imm434 iro1Δ/iro1Δ imm324/imm324 [17]) was grown to an optical density at 600 nm (OD600) of 0.8 in YPD media at 30°C in aerated flasks. Cells were harvested by centrifugation at 3,000 × g for 5 min, and the pellet was washed twice with phosphate-buffered saline (PBS) and resuspended in 400 μl of YPD media. Half of the \textit{C. albicans} cell suspension was used to inoculate aerated flasks containing fresh YPD media (normoxia), and the second half was added to bottles containing fresh YPD media flushed with nitrogen to remove oxygen (hypoxia). Different time points following this shift from normoxic to hypoxic growth conditions were considered (5, 10, 20, 30, 40, and 60 min). Cultures were harvested by centrifugation at 3,000 × g for 5 min, and the pellets were rapidly frozen in liquid nitrogen. For each time point, two biological replicates were performed. Mutant strains were treated similarly.

The antimycin A inhibition test was performed quantitatively using a Sunrise shaker/reader (Tecan) with continuous shaking at 564 rpm and replicates were performed. Mutant strains were treated similarly.

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The antimycin A inhibition test was performed quantitatively using a Sunrise shaker/reader (Tecan) with continuous shaking at 564 rpm and 30°C. Cells were grown in synthetic complete (SC) media, and OD605 readings were taken every 10 min. Antimycin A was added to reach a final concentration of 20 μg/ml. OD605 values during the exponential phase were used to calculate the doubling time of each mutant. The spot dilution assay was performed as follows: cells were serially diluted 10 times, plated on YPS medium (10/10/10/10 yeast extract, 20/20/20/20 polypeptide, 200/200 insoluble fast starch), and incubated in an anaerobic chamber at 30°C for 5 days. The chamber was flushed daily with nitrogen.

For the transcriptional profiling experiments, a total of two biological replicates were considered for each strain and each time points. Growth of the mutant strains \textit{Δsko1} (18), \textit{Δtye7} (10), \textit{Δabrl} (19), \textit{Δcerd} (20), and \textit{Δsitd} (21) for the transcriptional profiling experiment was performed as described for the SN148 strains for the hypoxic time course. The \textit{Δcerd}, \textit{Δtye7}, and \textit{Δabrl} mutants were assayed after 10, 30, 60, 90, and 120 min, while the \textit{Δsitd} and \textit{Δsko1} mutants were profiled at 10, 30, 60, and 90 min of hypoxia.

For gene expression profiling of cells treated with carbonyl cyanide 3-chlorophenyl hydrazone (CCCP; Sigma-Aldrich), saturated overnight cultures of wild-type (wt) \textit{C. albicans} SN148 were diluted to a starting optical density at 600 nm (OD600) of 0.1 in 100 ml fresh YPD media, grown at 30°C to an OD600 of 0.8, and then split into two 50-ml cultures. CCCP was added to the experimental culture to reach a final concentration of 50 μM. An equal volume of dimethyl sulfoxide used to dissolve CCCP powder was added to the control culture. Following a 10-min incubation, the cells were harvested by centrifugation and stored at −80°C.

**RNA preparation.** To extract RNA from cells, samples stored at −80°C were placed on ice and RNeasy buffer RLT was added to pellets at a ratio of 1:1 (vol/vol) of buffer/pellet. The pellet was allowed to thaw in the buffer and processed with a vortex device briefly at high speed. The resuspended pellet was placed back on ice and divided into 1-ml aliquots in 2-ml screw cap microcentrifuge tubes containing 0.6 ml of 3-mm-diameter acid-washed glass beads. Samples were homogenized 6 times for 1 min each time in a FastPrep-24 bead beater for 60 s at 6.5 m/s. Samples were placed on ice for 5 min after each homogenization step. Following homogenization, the Qiagen RNeasy protocol was followed as recommended. Total RNA samples were eluted in RNase-free H2O. RNA quality and integrity were assessed using an Agilent 2100 Bioanalyzer.

**Microarray hybridization and processing.** cDNA labeling and microarray production were performed as described by Nantel et al. (22). Briefly, 18 μg of total RNA was reverse transcribed using 9 ng of oligo(dt)18 in the presence of Cy3 or Cy5-dCTP (Invitrogen) and 400 U of Superscript III reverse transcriptase (Invitrogen). After cDNA synthesis, template RNA was degraded by adding 2.5 U of RNase H (Promega) and 1 μg RNase A (Pharmacia) following incubation for 15 min at 37°C. The labeled cDNAs were purified with a QiAquick PCR purification kit (Qiagen). Prior to hybridization, Cy3/Cy5-labeled cDNA was quantified using a NanoDrop ND-1000 UV light-visible light (UV-VIS) spectrophotometer (NanoDrop) to confirm dye incorporation. DNA microarrays were processed and analyzed as previously described (23). We obtained an average R square correlation of 0.805 between the log2 ratios of replicate microarray experiments.

**Functional analysis and modeling of transcriptional profiles.** The Gene Set Enrichment Analysis (GSEA) PreRanked tool was used to determine whether defined lists (or sets) of genes exhibited a statistically significant bias in their distribution within a ranked gene list (see http://www.broadinstitute.org/gsea/ for details) (24). This required the construction of an extensive gene set and annotation database using publicly available data, including 171 lists of up- and downregulated genes from microarray experiments, genes whose promoters contain 1 of 38 conserved TF-binding motifs (25), in vivo promoter targets derived from chromatin immunoprecipitation with microarray technology (ChIP-chip) experiments performed on 35 transcription factors, members of 3,601 Gene Ontology (GO) term categories, and 152 pathways, as curated by the Candida Genome Database (CGD) (26), and 4,777 protein-protein interaction complexes derived from \textit{Saccharomyces cerevisiae} data extracted from the \textit{Saccharomyces Genome Database} (SGD) (27) and BioGRID (28). Since profiles can exhibit correlations with hundreds of overlapping gene sets, GSEA results were further organized and visualized using the Cytoscape Enrichment Map plug-in (29). Modeling the temporal changes in TF activities (TFAs) was performed by Network Component Analysis (30). Calculations were performed in Matlab using the NCA algorithm downloaded from the web site http://www.seas.ucla.edu/~liaoj/. We used default values, and the number of cycles was set at 50. The node and edge model was constructed in Cytoscape 2.8.31 and laid out using the edge-weighted spring-embedded model. NCA–calculated Control Strength (CS) values (which represent the influence of a TF on each of its target promoters) were used to represent the spring strength; consequently, the value for the maximum edge weight to consider was set to 10. All other settings were left at their default values.
RESULTS

Temporal gene expression profiling of the C. albicans early adaptive response to hypoxia. We used DNA microarrays to measure the changes in transcript abundance that occur during the early adaptation of C. albicans to low oxygen concentrations. Modulated genes were identified by comparing the transcriptional profiles of cells exposed to a low oxygen concentration (less than 1%), at six different time points (5, 10, 20, 30, 40, and 60 min), to those of cells that continued to grow under normoxic conditions. As illustrated in Fig. 1, the resulting transcriptional response was extremely rapid, with highly significant changes in the abundance of hundreds of transcripts occurring within the earliest time point of 5 min. Using a 1.5-fold cutoff, 777 early-hypoxia-responsive genes were significantly modulated at the earliest time point (Fig. 1; see also Dataset S1 in the supplemental material). At the peak of the hypoxic response (20 to 30 min), we observed a drastic reorganization of the cells’ transcriptional profiles affecting 1,044 (17%) of the genes measured on our microarrays. The amplitude of the response then tapered off and stabilized into a profile similar to that which has been observed in steady-state experimental designs.

Functional investigation of hypoxia-regulated genes. We used Gene Set Enrichment Analysis (GSEA; see http://www.broadinstitute.org/gsea for details) to correlate hypoxic transcript profiles with C. albicans genome annotations and other transcriptional profiles. GSEA is a computational method that determines whether defined lists (or sets) of genes exhibit a statistically significant bias in their distribution within a ranked gene list (24). As it was initially developed to analyze data from mammalian genomes, its application to C. albicans data required the construction of a database of 8,852 gene sets using our own and publicly available data (see Dataset S2 and Materials and Methods for details). Since profiles can exhibit correlations with hundreds of overlapping gene sets, GSEA results were further organized and visualized using the Cytoscape Enrichment Map plug-in (29), which produces networks of gene sets with significant overlap. These networks, such as the one illustrated in Fig. S1 and Dataset S3 in the supplemental material, allow us to correlate biological functions to gene expression profiles and to identify correlations with transcripts that are modulated in response to another stimulus or to gene mutation or groups of genes whose promoters are recognized by a specific TF. Ultimately, these correlations were visualized using the Cytoscape Enrichment Map plug-in (29), which produces networks of gene sets with significant overlap. These networks, such as the one illustrated in Fig. S1 and Dataset S3 in the supplemental material, allow us to correlate biological functions to gene expression profiles and to identify correlations with transcripts that are modulated in response to another stimulus or to gene mutation or groups of genes whose promoters are recognized by a specific TF. Ultimately, these correlations were visualized using the Cytoscape Enrichment Map plug-in (29), which produces networks of gene sets with significant overlap.

In order to extract maximum information from the hypoxic transcriptome, transcripts exclusively specifying each time point were also determined and analyzed using GO annotation (see Fig. S2 and Dataset S4 in the supplemental material). The 5-min time point following oxygen depletion was characterized by a significant modulation of genes related to different components of transcription regulation such as TFs (CAP1, HMO1, RIM101, SKO1, UGA33, SFP1, and ROB1) and to components of the general transcription machinery (SSLI and MOT1) and the nucleosome re...
modeling complex (HIR3), suggesting a tight control of transcriptional regulatory circuits to acclimate its growth to the hypoxic environment. Transcript levels of many characterized TFs controlling different stress responses such as Cap1, Sko1, and Rim101 were upregulated, implying that hypoxia is probably perceived as a general stress. Endosome transport and cytoskeleton organization (complex nucleation of ARP2 and ARP3 [ARP2/3]) characterized early hypoxic adaptation at 10 min and 20 min, respec-

**FIG 2** Functional classification of the early hypoxic response. (A) General view of the functional roles of hypoxia-modulated genes as determined using GSEA and GO term analysis. Red and green bars indicate the time points at which the gene sets are significantly upregulated or downregulated. COA, coenzyme A. (B) GSEA graphs of four gene sets that were highly correlated with genes that were upregulated 20 min after the initiation of hypoxia. These genes were repressed in a Δcrr4 knockout strain (30), upregulated in a Δsit4 mutant under hyphal growth conditions (33), and upregulated in a Δsko1 mutant (31) and were those whose promoters are bound by the Tye7 transcription factor (10).
tively. Genes related to vacuolar organization and protein targeting of the endoplasmic reticulum (ER) were specifically activated late at 60 min.

**Contribution of the TFs Tye7 and Sko1, the phosphatase Sit4, and the transcriptional regulator Ccr4 to the early hypoxic transcriptional response.** Interestingly, GSEA revealed very strong correlations between the hypoxic transcriptional response and a variety of transcriptional profiles obtained from C. albicans microarrays over the past 11 years. Some were expected, such as profiles coming from studies aimed at measuring long-term adaptation to hypoxia (9–11) or from investigations where fungal cells were found under hypoxic conditions such as during biofilm formation (4) or following phagocytosis by immune cells (32, 33). In addition, several microarray experiments conducted in C. albicans were used to infer the effects of gene mutations, and many of the resulting profiles are highly similar to those observed in hypoxia. Examples illustrated in Fig. 2B include profiles from the cells missing the Ccr4 mRNA deadenylase (20), the Sko1 TF (19, 34), and the Sit4 phosphatase (21). The correlation between these profiles and the early hypoxic response is more significant than the correlation with the profiles from cells missing the TYE7 gene, which encodes a transcription factor whose role in mediating the transcriptional response to hypoxia has been clearly demonstrated (10, 16).

It is intriguing that the transcriptional profiles of Δsko1, Δccr4, and Δsit4 mutants growing under normoxic condition were similar to that of the early hypoxic response. A plausible explanation is that the three mutants have defective mitochondria leading to inefficient oxygen consumption or utilization, which in turn results in hypoxic growth even under normoxic conditions. Δccr4 and Δsit4 mutants, but not the Δsko1 mutant, were hypersensitive, compared to the wt strain, to antimycin A, an inhibitor of the mitochondrial respiratory chain (Fig. 3A). In accordance with the earlier hypothesis, this finding suggests that the mitochondria of these mutants are compromised, thus leading to inappropriate oxygen consumption. In a support of this assumption, Dagley et al. (20) demonstrated that mitochondria of the Δccr4 mutant are dysfunctional and have an abnormal morphology.

Additional microarray experiments were conducted in order to obtain a clearer picture of the effects of these regulators on the transcriptional response to hypoxia. We also profiled the hypoxic response in Δtye7 mutants as well as in cells missing the Ahr1 transcription factor, which has been shown previously to bind to and to modulate the Tye7 gene promoter (19). Mutants Δccr4 and Δsit4 were also taken into consideration to assess the effect of the mitochondrial defect on hypoxia sensing and adaptation. Preliminary quantitative PCR (qPCR) and microarray experiments suggested that all five mutants responded strongly to oxygen removal but that the speed of this response was slightly delayed compared to that seen with wild-type cells. Consequently, the Δccr4, Δtye7, and Δahr1 mutants were assayed after 10, 30, 60, 90, and 120 min whereas the Δsit4 and Δsko1 mutants were profiled at 10, 30, 60, and 90 min. We selected 1,016 genes whose transcript abundance changed by more than 2-fold under at least 4 conditions and organized the resulting profiles by hierarchical clustering. As seen in Fig. 1, although most mutants responded in apparently similar manners to oxygen removal, the timing and the amplitude of the responses were diminished compared to wild-type cell results. We used GSEA and Welsh t tests to define the functional gene categories whose change in transcript abundance was the most affected in the various mutants. As illustrated in Fig. 4, all five regulators appeared to control distinct but overlapping functional gene groups, with the exception of Ahr1, which did not appear to control any functional groups outside those regulated by Tye7. Deletion of the TYE7 gene had by far the greatest impact, with 1,103 genes showing significant differences in their transcript abundance compared to the response observed in wild-type controls. Our results to date illustrate the difficulty is clearly assigning distinct regulators to specific functions within what is obviously a complex and highly redundant regulatory system.

We performed a variety of experiments aimed at determining if Δccr4, Δsko1, and Δsit4 cells are defective in their ability to grow in hypoxia. Growth curves in different liquid media, including Spider, YPD, YPS, RPMI, and synthetic complete (SC) media, failed to show significant growth defects in the absence of oxygen (results not shown). On solid media, with the exception of the Δccr4 mutant, which showed a significant growth defect specifically on YPS but not on YPD, SC, Spider, or RPMI media, growth of the Δsko1 and Δsit4 mutants was not impaired (Fig. 3B). While the Δtye7 mutant did exhibit reduced growth on solid media as pre-
vously reported (10), it did not show any impairment in its doubling time in liquid cultures compared to wild-type controls.

**Modeling the transcription factor network and its response to hypoxia.** Hierarchical clustering and GSEA were helpful only partially in understanding the roles of TFs in mediating the hypoxic response. While genes whose promoters were recognized by Ty7 and Upc2 were well represented in the lists of upregulated genes, many of them were known targets of multiple TFs and a precise pattern was difficult to elucidate. As shown in Table 1, genes whose promoters were recognized by multiple known TFs were more likely to be modulated in response to hypoxia. To decipher the transcription factor networks (TFN) controlling this response, we used Network Component Analysis (NCA) for the challenging task of linking large TF-binding data sets with time-response, we used Network Component Analysis (NCA) for the time-dependent changes in TF activity which can result from changes in protein levels, intracellular localization, or posttranslational modification. The NCA algorithm also calculates a Control Strength (CS) value for each TF-promoter pair that reflects the relative influences (positive or negative) of the TF on the expression of each of its individual target genes. Initial and computed TFA and CS matrices are included in Dataset S5.

A precondition of the NCA algorithm is that it can model only a number of TFs equal to the number of microarray experiments minus 1. While our microarray data set included data on 30 different experiments, fortuitously, at the time of this analysis (in January 2013), we had access to promoter occupancy data on 30 C. albicans TFs. The list of TFs included in this analysis and the origins of their in vivo promoter targets are included in Table 2. Following preliminary model runs, we decided to exclude Ndt80 from the NCA because its high number of promoter targets (>2,500) gave it an overly important influence that was not supported by the phenotypes of the Δndt80 mutant (23, 54). In addition, inclusion of Ndt80 significantly increased the standard deviation of the calculated TFA values between distinct model runs and resulted in a poorer separation of up- and downregulated genes in the edge and node models of the TF network. The model thereby includes transcriptional profiling data on 3,145 genes whose promoters were identified as an in vivo target for at least one of the 29 TFs.

Figure 5 shows the calculated TFA values (in blue) for 16 of the most interesting TFs (TFAs for all 29 are shown in Fig. S3 in the supplemental material). These are superimposed with mRNA transcript levels (in red) for each TF gene. As much as possible, all graphs were kept to the same scale, thus permitting us to observe which TF had the greatest changes in activity in response to hypoxia. Interestingly, many TFs showed a remarkable correlation

| No. of bound TFs | No. of genes | 2-Fold change in hypoxia data set (%) | 2-Fold change in hypoxia data set (%) | Hypergeometric test P value |
|-----------------|-------------|-------------------------------------|-------------------------------------|---------------------------|
| >10             | 15          | 8                                   | 53.3                                | 0.005                     |
| 7–9             | 103         | 54                                  | 52.4                                | <1 × 10^{-10}             |
| 4–6             | 477         | 187                                 | 39.2                                | <1 × 10^{-10}             |
| 3               | 339         | 126                                 | 37.2                                | <1 × 10^{-10}             |
| 2               | 620         | 213                                 | 34.2                                | <1 × 10^{-10}             |
| 1               | 1,449       | 390                                 | 26.9                                | <1 × 10^{-10}             |
| 0               | 3,815       | 38                                  | 1                                   | <1 × 10^{-10}             |
| Total           | 6,806       | 1,016                               | 14                                  |                           |
between their TFA and mRNA abundance profiles, which suggests that their activities are mostly modulated through changes in their gene expression levels. These highly correlated TFs include Tye7, Cbf1, Cph1, Efg1, Hap43, Mcm1, Rap1, Sfu1, Tact1, and Upc2. Other TFs, such as Ada2, Ahr1, and Mrr1, showed significant distinctions between their TFA values and mRNA expression levels suggestive of a more complex mechanism of regulation. Many TFs, such as Cap1, Csr1, Tac1, and others, showed little change in TFA during the hypoxia time courses, suggesting that they play only a minor role in this process. This representation is also useful in determining how the TF networks react in the context of a gene-deleted strain. For example, the timing and amplitude of the increase in Tye7 TFA were markedly diminished in all five of the Δccr4, Δsit4, Δsko1, and Δahr1 mutants, which may explain part of the delayed transcriptional response. This cannot be attributed to a global delay in the transcriptional to hypoxia, since the activity of Upc2 was not significantly impacted in the Δahr1, Δccr4, Δsit4, and Δsko1 mutants even though it bound to the promoters of a large number of hypoxia-activated genes. Conversely, the activities of Ada2 and Upc2 are markedly reduced in Δtye7 mutants. One possible explanation is that these TFs require the presence of Tye7 to exert their influence of target genes, a hypothesis that is likely correct for the Ada2 coactivator (39). The Upc2 gene promoter is also an in vivo target for Tye7 (10), and these results suggest that upregulation of Upc2 gene transcription by Tye7 is an important step in the hypoxic response. Finally, this type of analysis can also reveal unexpected correlations that could form the basis for future hypotheses and studies, such as the absence of a strong hypoxia-dependent reduction in the activity of the iron metabolism TF Sfu1 in the absence of the Sit4 phosphatase or the significant reductions in the Tec1 transcript and activity levels in Δahr1 mutants.

Visualization of the transcription factor network. We used Cytoscape (http://www.cytoscape.org) to build a node and edge model of the C. albicans TF network. We selected 1,041 genes that were included in the NCA model and that also showed at least a 2-fold change in transcript abundance under at least 4 of the strain and time point combinations assayed in the microarray experiments. We also removed the Ifh1 TF since it bound to the same list of 2-fold-modulated gene promoters as Fhl1. The layout of the network was produced using an edge-weighted spring embedded algorithm that integrates the NCA-calculated CS values as the spring strength. As illustrated in the small example in Fig. S4 in the supplemental material, such an algorithm has a tendency to position gene nodes in closer proximity to the TFs that have the strongest influence on their mRNA levels. Once the nodes were colored using transcriptional profiling data, as illustrated in Fig. 6 and Movie S1 in the supplemental material, such a network showed a notable ability to separate hypoxia-upregulated and -downregulated genes. This effect is also very obvious if the nodes are colored according to their rates of change between the various time points, as illustrated in Fig. S5 and in Movie S2 in the supplemental material. Coloring using rates of change also results in a better correlation between the TF nodes and their immediate environment. To ensure that our results are not simply the consequence of selecting the most highly modulated genes, we repeated the TF network construction using all 3,141 genes that were targeted by at least one of the 28 TFs. As shown in Fig. S6 in the supplemental material, the resulting model can still separate most of the upregulated and downregulated genes to distinct regions, although the effect is not as clear-cut as in Fig. 6.

Visualization of transcription factor redundancy. The TF network model is also useful to illustrate the level of TF redundancy that can be harnessed to control this important aspect of C. albicans physiology. No single TF was found to control a majority of either the upregulated or downregulated gene promoters. Nevertheless, combining multiple regulons has proved to be informative. As shown in Fig. 7, a union of regulons of Mrr1, Tye7, and Upc2 includes 215/380 (56.6%) of genes whose transcript abundance increased by at least 2-fold during early hypoxia. Of 151 Tye7 promoter targets, 66 were also recognized by Mrr1 and/or Upc2 whereas 11 gene promoters were recognized by all three TFs. The proportion of upregulated gene promoters increases to 72.6% if we include the Cbf1 regulon and 80.8% upon the addition of Hmo1 targets. The situation is more complex for downregulated genes since targets for most of the remaining TFs include a mixture of downregulated and upregulated promoters. Nevertheless, selecting the combined regulons of Cbf1, Efg1, and Mcm1 and then excluding from this list members of the Mrr1/Tye7/Upc2 regulon selected 80.0% of significantly downregulated genes (Fig. 7).

In addition to network redundancy, part of the challenge in interpreting the effect of TF gene mutations is the high level of linkages between TFs and TF gene promoters. As shown in Fig. S7 in the supplemental material, the 27 TFs have a total of 128 TF-
promoter interactions among them. On average, each TF gene is potentially regulated by 4.7 known TFs. Nevertheless, the effects of the Δtye7 and Δahr1 gene deletions can be visualized in the context of TF networks. The subnetworks illustrated in Fig. 8 include members of the Tye7 and Ahr1 regulons whose expression is modulated in early hypoxia as well as the other TFs that interact with the regulon gene promoters. Of note, the promoters of 128 of the 151 (84.8%) hypoxia-regulated members of the Tye7 regulon are bound by at least one other known TF. In addition, most of the 23 hypoxia-responsive genes whose promoters are bound by Tye7 alone (12 o’clock position in the cluster) are not modulated by hypoxia in a Δtye7 mutant, which is consistent with their reduced regulatory redundancy. As for Ahr1, 73 of 75 (97.3%) of its hypoxia-regulated targets are also recognized by other TFs. Nevertheless, the impact of a Δtye7 gene deletion on the upregulation of its gene targets is fairly significant, with many of its target genes exhibiting reduced induction levels in response to hypoxia. The effects of Δahr1 deletion are also evident albeit different in nature. Many Ahr1 targets show reduced expression in response to hypoxia, especially genes that encode other TFs (see also Fig. S7 in the supplemental material). The Ahr1 regulon subnetworks also help to explain the model of partial hierarchical regulation between Ahr1 and Tye7 that is apparent in Fig. 4. While both regulate genes associated with carbohydrate metabolism and protein production, there are nevertheless several Tye7-independent genes whose expression is significantly impaired in the Δahr1 mutant, although they do not fall into a well-defined functional classification. This could be explained by the fact that, in addition to TYE7, at least
four other TF gene promoters (BRG1, EFG1, MRR1, and ROB1) are recognized by Ahr1.

The uncoupling agent CCCP partially mimics the early hypoxic transcriptional response. Previous works have shown that many fungi, including S. cerevisiae, Schizosaccharomyces pombe, Cryptococcus neoformans, and C. albicans, sense oxygen depletion by gauging the cellular level of metabolites requiring oxygen for their biosynthesis such as ergosterol, heme, and unsaturated fatty acids (1, 13). In our study, activation of genes involved in oxygen-dependent metabolism, including ergosterol and heme, reflecting a compensatory feedback mechanism to supply the cell for the depleted metabolites, occurred late in the process (5-40 min) (Fig. 2A). Consequently, it is likely that the sterol or heme depletion cues are not associated with the early transcriptional response to hypoxia, which occurred remarkably quickly (5 min). Since hypoxia results in a drop of energy production as a consequence of the reduction of the ATP-generating activity of oxidative phosphorylation, we hypothesized that a sudden depletion in ATP might serve as a cue of hypoxia perception in C. albicans.

In order to assess if ATP depletion is the primary cue to sense hypoxia, we used the uncoupler compound carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which abolishes the linkage between the respiratory chain and oxidative phosphorylation (35). We compared the transcriptional profiles of wild-type cells exposed to CCCP for 10 min to that of untreated cells. Scatter plots in Fig. 9A show that the resulting transcriptional profile is very similar to the profiles obtained after 10 to 20 min of hypoxia. Comparative GSEA of the hypoxia 10-min time point also shows that CCCP and loss of oxygen have very similar effects at the functional level. CCCP leads to a faster reduction of genes whose products participate in the oxidative phosphorylation chain, the process targeted by the drug. In addition, CCCP treatment has been shown to result in elevated reactive oxygen species (ROS) production (56), which would explain the resulting upregulation of genes implicated in the oxidative stress response. These results support the hypothesis that rapid reduction of ATP synthesis mimics the rapid removal of oxygen.

The transcriptional profiles of cells treated with CCCP for 10 min were included in the NCA model. As seen in Fig. 5, most TFs showed changes in their calculated TFAs that were similar to their activity changes during early hypoxia. Exceptions include Sfu1, which showed an increase in both transcript levels and TFA, and...
Upc2, which showed no increase in TFA even though its transcript levels increased more than 8-fold. The most notable results were the CCCP-dependent TFA increases in three TFs that have been implicated in drug responses: Cap1, Mrr1, and Tac1 (42, 51, 53, 57). The responses of Cap1 and Tac1 are unique to CCCP, NCA modeling having suggested that these did contribute significantly to the early hypoxic response. As shown in Fig. 10, these increases in Cap1/Mrr1/Tac1 activity have significant consequences for the transcript abundance of their respective regulons. This representation also suggests that most of the differences between the transcriptional responses to hypoxia and CCCP treatment can be attributed to the increased activity of these 3 TFs.

**DISCUSSION**

The facultative anaerobic lifestyle of the opportunistic yeast *C. albicans* implies growth in both oxygen-rich environments such as cutaneous epithelial cells and oral mucosa and oxygen-poor niches such as internal organs or even the intracellular environment such as hepatocytes or immune cells (1, 58). This suppleness requires an effective metabolic flexibility and a tight regulation of the switch between fermentation and full cellular respiration within different niches inside the host. In contrast to the model yeast *S. cerevisiae*, which ferment sugars to ethanol even under aerobic conditions, *C. albicans* oxidizes carbohydrates through respiration under aerobic conditions and ferments carbohydrates under hypoxic conditions (8). Thus, *C. albicans* is an attractive eukaryotic model to study metabolic plasticity and growth adaptation in response to oxygen fluctuations. Despite the physiological and the biological relevance of hypoxia in *C. albicans* biology as well as in other human fungal pathogens, there is little information on how this fungus adapts, grows, and infects host cells in oxygen-poor niches. Many questions of biological significance remain to be answered. How does *C. albicans* sense hypoxia? What are the adaptive mechanisms associated with a short or prolonged period of hypoxia? What are the signaling pathways and TF networks that mediate hypoxic adaptation and hypoxic filamentation? How does hypoxia affect other developmental stages such as biofilm, chlamydospores, and opaque cells?

The majority of studies investigating the transcriptional response to O2 depletion did not investigate the temporal dynamics of the transcriptome and were undertaken at later time points that reflect a steady-state adaptation to hypoxia (9–11). In this work, we have elucidated the transcriptional program accompanying the early adaptive response to hypoxia across a comprehensive time course. We have also illustrated the contribution of different regulators controlling the early adaptive response and uncovered considerable complexity and redundancy within the hypoxic-response networks.

**Temporal dynamics of *C. albicans* hypoxic transcriptome.** For the first time in a fungus, our work provides a comprehensive and dynamic portrait of the different O2-controlled biological processes accompanying the immediate adaptation to hypoxia. The response of *C. albicans* to a sudden depletion of oxygen was extremely rapid, with significant changes in gene expression occurring within the first 5 min (777 transcripts differentially expressed). The transcriptional response of *C. albicans* to hypoxia had been well elucidated in earlier studies where the transcriptome was assessed by considering a single time point. Setiadi et al. (9) reported that, upon exposure to low oxygen levels for more than 120 min, *C. albicans* upregulated transcripts related to ERG biosynthesis, fatty acid and iron metabolism, cell wall structure, glycolysis, fermentation, and translation, while genes of the tricarboxylic acid (TCA) cycle and respiration were repressed. Recently, Synnott et al. (11) described similar findings where cells growing under hypoxic conditions for 3.5 h generally exhibited the same core transcriptional response. Based on our GSEA (Fig. 2A), these
findings are very similar to our transcriptional profiles obtained after 40 to 60 min or even 24 h (data not shown), suggesting that the previous studies reported a transcriptional pattern related to a steady-state hypoxic condition.

The early hypoxic transcriptional wave was characterized by the repression of genes related to both cytosolic and mitochondrial translation in addition to genes required for the processing and the maturation of rRNA. Since production of ribosomes accounts for 70% to 80% of the total nuclear transcriptional capacity of eukaryotic cells (37), repression of this category of genes reflects that *C. albicans* cells are adjusting their growth rate and their energy expenditure in response to hypoxia. Interestingly, GSEA also uncovered a significant correlation between hypoxia-responsive genes and transcripts that were misregulated in the *Η9004 sit4* mutant.

In *S. cerevisiae*, the type 2A-related Sit4 serine-threonine phosphatase is a critical effector of the TOR (Target Of Rapamycin) signaling pathway, which couples nutrient availability to cell growth (59). Interestingly, many biological functions repressed during early hypoxic adaptation, such as nitrogen metabolism, translation, and amino acid metabolism, are the bona fide target functions modulated by the TOR pathway in different organisms, including *C. albicans* (60). Taken together, these observations reflect the potential contribution of the TOR pathway in metabolic control and energy homeostasis during the early hypoxic adaptation. Future works could investigate whether the TOR pathway, together with Sit4, plays a key role in signaling networks controlling hypoxic perception and adaptation in *C. albicans*.

**Transcriptional regulatory networks controlling hypoxic adaptation.** Beyond a fragmentary picture of hypoxia biology in fungi, very little is known regarding the TF networks that control hypoxic adaptation and growth. The complex transcriptional response to hypoxia and the large number of differentially expressed genes at each time point make the interpretation of the hypoxia-adaptive transcriptome particularly challenging. In an attempt to
mine the results of the current investigation and build a hypoxic TF network, we have integrated available ChIP-chip and ChIP sequencing (ChIP-Seq) data with the hypoxic expression profiles using the NCA algorithm to link the activity and the influence of TFs to the time-dependent changes in the mRNA abundance of their target genes. The NCA model confirmed that Tye7 plays a critical role in the activation of transcripts of the early hypoxic transcriptional wave (5 to 30 min). NCA uncovered a peak of Tye7 activity concomitantly with glycolytic and carbohydrate gene activation, confirming the key role of this TF in sugar metabolism (10). NCA was also very useful in assessing the interdependence of TFA among the transcriptional regulators considered for this work. In the case of the transcriptional coactivator Ada2, its TFA upregulation was completely abolished in mutant \( /H9004:\) tye7. This finding supports previous investigations where Ada2, a component of the histone acetylation complex SAGA, is recruited by other TFs to mediate acetylation and gene expression activation of target genes (47). This emphasizes the essential role of Tye7 in the combinatorial regulation of glycolysis by the transcriptional complex Ada2-Tye7.

Node and edge models are commonly used to illustrate TF networks. In \textit{C. albicans} research, they have been applied to mechanisms that control the white-opaque transition (50, 61), biofilm formation (41), iron metabolism (48), and host-pathogen interactions (62). Even though they were constructed from a limited number of TFs, they nevertheless resulted in complex, interlocking networks with redundant connections and an even greater level of connectivity between TFs and TF gene promoters. In addition, these networks were usually produced from ChIP-chip and ChIP-Seq data obtained from cells that had been grown under conditions that were relevant to the mechanisms under study. The TF network constructed in this study is different in many ways. (i) Since we did not have the resources necessary to build a large hypoxia-specific promoter occupancy data set, we instead combined all ChIP-chip and ChIP-Seq data published by \textit{Candida} researchers prior to January 2013. This produced a much more interconnected network whereby 588 of 1,041 genes (56%) were connected to 2 or more TFs. (ii) We then integrated transcriptional profiling data in an NCA model to produce estimates of the influence of each TF on each of the corresponding target gene promoters. As illustrated in Fig. S4 in the supplemental material, these were then integrated in the layout of the TF network. (iii) Finally, we used transcriptional profiling data to color the nodes in the network. This representation demonstrated that, even though we used promoter occupancy data from a variety of sources, the distribution of genes within our network was not random but actually exhibited a spatial separation between the upregulated and downregulated genes.

**FIG 9** Transcriptional response to the CCCP uncoupling agent. (A) Scatter plots comparing the transcriptional responses of wild-type cells to a 10-min CCCP treatment to the hypoxic responses after 10 or 20 min. (B) Characterization of functional gene sets was achieved by combining GSEA results from the CCCP and a combination of the 10-to-20-min hypoxia profiles in an enrichment map. LSU, large subunit of ribosome.
One of the major findings of this work is the remarkable complexity and the redundancy of the hypoxic transcriptional regulatory network. Among transcriptional regulators uncovered by the NCA or GSEA as being essential for the expression of the early hypoxic transcriptional program, only a few (Δtyc7 and Δccr4 mutations) resulted in a growth defect under hypoxic conditions. This suggests that *C. albicans* cells exploit redundancy as a way to provide alternative failure-tolerant mechanisms that confer robustness to the hypoxic-response network. The origin of such robustness can be attributed to the fact that the promoters of a large proportion of the genes expressed during the time course were recognized by more than one TF (Table 2), implying that inactivation of a regulator might be buffered by the activity of other regulators. The robustness of the hypoxic response can be also attributed to the high level of linkages between TFs and TF gene promoters. In fact, 27 TFs have a total of 128 TF-promoter interactions, including a self-promoter occupancy, suggesting both feedback loop control and autoregulation. The complexity of the transcriptional regulatory network and the interconnectedness of transcriptional regulators in *C. albicans* were also emphasized in other circuits (41, 48, 50, 61, 62). As was hypothesized in these investigations, the robustness of the hypoxic-response network might be related to the fact that these circuits integrate different cues encountered in specific niches inside the host. The hypoxic circuits are most likely solicited in different niches where oxygen concentrations are fluctuating (1) and also in

FIG 10 Effect of CCCP treatment on the regulons of TFs associated with drug responses. Starting from the TF network structure in Fig. 6 (left column), we produced a subnetwork containing nodes with a direct connection to Cap1, Tac1, or Upc2 (right column). Nodes were colored according to their changes in transcript level after 20 min of hypoxia or a 10-min treatment with CCCP.
some growth state such as biofilm where fungal cells are confronting hypoxia.

How does C. albicans sense oxygen depletion? To confirm whether ergosterol depletion acts as a cue of hypoxic response and adaptation in C. albicans, Synnott et al. (11) compared their hypoxic transcriptional profiles to that of Δupc2 mutant cells or to that of cells treated with ketoconazole, the inhibitor of sterol biosynthesis. Their results demonstrated that ergosterol depletion mimicked partially the hypoxic response, where only hypoxia-induced genes coding for members of the CFEM family (RB75 and PAG7), ERG genes (ERG3 and ERG11), and genes required for iron assimilation (FRP1 and FET34) were upregulated following the exposure to ketoconazole. In this study, we found that the early hypoxic transcriptional response (10 to 20 min) was very similar to that expressed by cells treated by the uncoupling agent CCCP, which abolished the linkage between the respiratory chain and oxidative phosphorylation and consequently decreased the ATP amount in the cell. This result suggests that the drop in the ATP-generating activity of oxidative phosphorylation mimics the rapid removal of oxygen and emphasizes the role of mitochondria in hypoxia sensing and adaptation. This finding is further strengthened by the fact that mutants with defective mitochondria such as strains Δmtt4 and Δaccr4 were not able to respond quickly and effectively to oxygen depletion. Consistent with the later statement, the role of mitochondria, especially mitochondrial complex III, was shown to be essential for oxygen sensing and the activation of downstream hypoxic effectors in human cells (63).

We also observed that activation of genes involved in ergosterol and heme metabolism, reflecting a compensatory feedback to supply the cell with the depleted metabolites, occurred late in the process (>40 min). Consequently, the current model suggesting that hypoxia is sensed through the depletion of oxygen-dependent metabolites does not explain the rapid hypoxic transcriptional response. However, this does not exclude the possibility that hypoxia sensing is a complex phenomenon and that ATP diminution and drops in the level of metabolites such as ergosterol, heme, and unsaturated fatty acids are separate hypoxia cues operating on different time scales.

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