Zinc Cluster Transcription Factors Alter Virulence in Candida albicans

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ABSTRACT Almost all humans are colonized with *Candida albicans*. However, in immunocompromised individuals, this benign commensal organism becomes a serious, life-threatening pathogen. Here, we describe and analyze the regulatory networks that modulate innate responses in the host niches. We identified Zcf15 and Zcf29, two Zinc Cluster transcription Factors (ZCF) that are required for *C. albicans* virulence. Previous sequence analysis of clinical C. *albicans* isolates from immunocompromised patients indicates that both *ZCF* genes diverged during clonal evolution. Using *in vivo* animal models, *ex vivo* cell culture methods, and *in vitro* sensitivity assays, we demonstrate that knockout mutants of both *ZCF15* and *ZCF29* are hypersensitive to reactive oxygen species (ROS), suggesting they help neutralize the host-derived ROS produced by phagocytes, as well as establish a sustained infection *in vivo*. Transcriptomic analysis of mutants under resting conditions where cells were not experiencing oxidative stress revealed a large network that control macro and micronutrient homeostasis, which likely contributes to overall pathogen fitness in host niches. Under oxidative stress, both transcription factors regulate a separate set of genes involved in detoxification of ROS and down-regulating ribosome biogenesis. ChIP-seq analysis, which reveals vastly different binding partners for each transcription factor (TF) before and after oxidative stress, further confirms these results. Furthermore, the absence of a dominant binding motif likely facilitates their mobility, and supports the notion that they represent a recent expansion of the ZCF family in the pathogenic *Candida* species. Our analyses provide a framework for understanding new aspects of the interface between *C. albicans* and host defense response, and extends our understanding of how complex cell behaviors are linked to the evolution of TFs.

KEYWORDS Candida albicans; ZCF transcription factors; clonal evolution; gene duplication and expansion; virulence; host interactions

CANDIDA albicans is a fungal commensal that can cause infections ranging from persistent superficial infections to life-threatening systemic infections. In the last two decades, the pathogenic basis of *C. albicans* has been extensively studied (Mayer *et al.* 2013), highlighting molecular mechanisms and fitness costs that facilitate this commensal to become a life-threatening pathogen.

The human host harbors a diverse collection of microbial species that compete for resources, space, and nutrients. For species such as *C. albicans* that can switch from commensal to

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pathogenic growth, host adaptation depends critically on factors affecting growth rate. If growth is restricted, *C. albicans* will typically lose out in competition to microbes that divert host resources for their own reproduction. *C. albicans* must access macronutrients, such as carbon and nitrogen, and micronutrients, such as iron, to sustain growth. Typically, if pathogen growth cannot be controlled, then the infection persists and the host suffers from increased pathogen load. Thus, pathogen growth is the end result of an intricate interaction between the host and pathogen, as well as other species in the microbiome. Here, we describe mechanisms that *C. albicans* has evolved to integrate host-derived cues and direct cellular resources to manage such nutritional needs.

The dimorphic lifestyle of *C. albicans* requires regulation at the genetic level to ensure coordinated expression of genes. Transcription factors (TFs) play a key role in determining

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how cells function and respond to different environments, and ~4% of *C. albicans* transcripts code for TFs (Homann *et al.* 2009), the single largest family of proteins. TFs in *C. albicans* coordinate essential cellular functions, including biofilm formation (Nobile *et al.* 2009) and drug resistance (Cowen *et al.* 2002), as well as the transition from a commensal to a pathogenic lifestyle (Liu 2001). The zincfinger transcription factors are enriched in pathogenic *Candida* species, and show accelerated rates of evolution (Butler *et al.* 2009), suggesting they play key roles in recent adaptation.

The Zinc Cluster Family (ZCF) TFs represent a family of 82 Zn(II)₂Cys₆ DNA-binding proteins, and are restricted to the fungal kingdom (Schillig and Morschhauser 2013). A subset of 35 ZCFs are expanded through duplication and diversification in fungi capable of a pathogenic lifestyle, and are missing from rare pathogens and the nonpathogenic yeasts (Figure 1A; Butler et al. 2009). This suggests that this subset of ZCF transcription factors may contribute to the evolution of a pathogenic lifestyle. In addition, ongoing nonsynonymous mutations in ZCF genes were detected by analyzing sequence of C. albicans isolates from infected AIDS patients who are prone to active infection and require long-term fluconazole treatment (Ford et al. 2015). While large-scale phenotypic characterizations have highlighted the importance of ZCFs (Homann et al. 2009; Vandeputte et al. 2011), the specific functions of most family members remain unknown. Therefore, researchers must evaluate the function of these TFs one at a time (Bohm et al. 2016).

To gain a better understanding of how ZCFs mediate host interaction, we focused this study on two ZCF genes that are conserved in pathogenic Candida species, but not in nonpathogens (Figure 1, A-C). ZCF15 is specifically expanded in pathogenic species, with three paralogs in C. albicans (Supplemental Material, Figure S1), and in each of the pathogenic Candida, but absent in related nonpathogenic species. This is the highest count among the ZCF genes found in pathogens. Similarly, ZCF29 is present as a single ortholog in other pathogenic yeasts (C. tropicalis, C. parapsilosis, C. guilliermondii, and C. lusitaniae), but is not present in phylogenetically related nonpathogenic species (Saccharomyces cerevisiae, S. paradoxus, or S. castelli) (Figure 1). Both ZCF15 and ZCF29 appear to be under strong purifying selection $(d_N/d_S \text{ of } 0.125)$ and 0.127, respectively) compared to their closest ortholog in C. dubliniensis, indicative of conserved function in Candida. However, these genes also appear variable between C. albicans isolates, with nonsynonymous changes observed between serial isolates from patients (Ford et al. 2015), so could potentially vary in function or specificity between isolates.

We employed experimentally tractable *in vivo* and *ex vivo* models to elucidate the role of these ZCF TFs in the innate immune system (Jain *et al.* 2009, 2013). Among the 35 ZCFs, these mutants exhibit similar redox sensitivity profiles in phagocytes *ex vivo*, and nematodes *in vivo*. Null mutations in each gene result in sensitivity to reactive oxygen species

in vitro. To understand the regulation mediated by *Zcf15* and *Zcf29*, we measured the transcriptional response to genetic and environmental perturbations, thereby deciphering their genetic and molecular networks. The biological significance of these networks was confirmed by the identification of downstream genes they regulate specifically in response to reactive oxygen species (ROS) that are typically produced by host innate immune defenses.

Materials and Methods

Strains and media

All *C. albicans* strains used were obtained from the Fungal Genetic Stock Center (FGSC), and are described in Table S1. *C. albicans* deletions in *ZCF15*, *ZCF29*, and their isogenic wild type were obtained from the FGSC Suzanne Noble knockout set, and their genotypes are described in Table S1.

To complement *ZCF15* deletion, we reintegrated *ZCF15* using gap repair cloning in *S. cerevisiae* as described in Gerami-Nejad *et al.* (2013). Briefly, *ZCF15* ORF $\pm \sim$ 650 bp was PCR amplified and cotransformed in *S. cerevisiae* BY4747 with *Bmg*BI-digested pSN105 (Noble *et al.* 2010). Plasmids recovered from URA⁺ transformants were verified for the presence of *ZCF15* by both PCR and diagnostic restriction digestion. The plasmid was subsequently cut with *Pme*I, and transformed in *zcf15/zcf15* using standard lithium-acetate protocols. Proper insertion of the *ZCF15-ARG4* insertion cassette was confirmed by checking the presence of the new 5' and 3' junctions in the *LEU2* locus.

ZCF sequence analysis

Analysis of C. albicans genomes (Schillig and Morschhauser 2013) identified 80 TFs with a Zn(II)2Cys6 motif known to regulate cellular processes including multi-drug resistance, cell wall architecture, regulation of invasive filamentous growth, and other nutritional cues (Maicas et al. 2005). Approximately a third of these TFs are poorly characterized, and retain the generic ZCF designation. The Fungal Orthogroups tool within the Candida Genome Database was used to identify orthologs and paralogs of ZCFs (Figure 1, B and C). To evaluate selective pressure on ZCF15 and ZCF29, the most closely related gene in C. dubliniensis was mapped using the Candida Gene Order Browser (Maguire et al. 2013); C. tropicalis orthologs were not used, as rates of synonymous substitution appear saturated ($d_S > 6$ for both genes). Protein sequences were aligned using MUSCLE (Edgar 2004), and converted to codon alignments with PAL2NAL (Suyama et al. 2006); d_N/d_S ratios were calculated with Codeml (Yang 2007). For phylogenetic analysis of ZCF15 or ZCF29 with related orthologs (Wapinski et al. 2010), protein sequences were aligned with MUSCLE (Edgar 2004), and gap regions removed with TrimAl (Capella-Gutierrez et al. 2009). The best model was identified with Protest v3.4 (Darriba et al. 2011) (PROTGAMMAJTT for ZCF15 and PROTGAMMALG for ZCF29), and used by



Figure 1 Conservation of ZCF TFs, and identification of two sensitive to oxidative stress. (A) The *C. albicans* genome encodes for ~240 TFs; 82 listed in the figure belong to the special class of Zn(II)₂Cys₆ DNA binding proteins that are restricted to the fungal kingdom (not present in humans or plants), and expanded in pathogens. Of these, 35 are clustered (on the right) by phylogenetic relatedness. These ZCFs are poorly characterized and do not have homologs in other fungi. They are novel and of unknown function. *ZCF15* and *ZCF29* represent two extremes of the expansion spectrum; *ZCF15* has three other paralogs, and *ZCF29* has none. Phylogenetic analysis of (B) *ZCF15* and (C) *ZCF29* in *Candida* and related fungi. Orthologs of *ZCF15* and *ZCF29* in *Candida* and related fungi were previously identified (Wapinski *et al.* 2010). Phylogenies inferred with RAxML from aligned protein sequences are shown with the fraction of 1000 bootstrap replicates supporting each node. Genes shown in the tree correspond to species as follows: CTRG, *C. tropicalis*; CPAG, *C. parapsilosis*; LELG, *L. elongisporus*; PGUG, *P. guilliermondii*; CLUG, *C. lusitaniae*; DEHA, *D. hansenii*; AN, *A. nidulans*.

RAxML v7.7.8 (Stamatakis 2006) to infer the phylogenetic relationship of each *ZCF* gene.

Reverse genetic screen and in vivo virulence assays

In order to identify novel *C. albicans* virulence determinants, we screened a collection of 724 *C. albicans* mutants (~12% of the genome) for their abilities to induce the Dar phenotype (Jain *et al.* 2009) in *Caenorhabditis elegans*. Live nematodes were infected with *C. albicans* to identify mutants that exhibited altered phenotype in the worms. The assays have previously been described (Jain *et al.* 2013). Briefly, 30 healthy *C. elegans* were exposed to *C. albicans*, which was mixed into their diet (Jain *et al.* 2009). The percentage of worms showing signs of infection (Jain *et al.* 2013) were scored manually 4 days post infection. To generate survival curves, 20 young synchronized *C. elegans* N2 were exposed to

the infection diet. Worms were scored as live or dead daily by gentle prodding with a platinum wire. Dead worms were discarded, while live ones were transferred to new infection plates. Worms accidentally killed while transferring, or found dead on the edges of the plates, were excluded from further analysis. For data analysis, SPSS (IBM) was used to generate Kaplan-Meier survival curves. Each worm that died on the plate was entered as a "1," indicating the event of death due to fungal disease took place. Worms that were found dead on the rim of the plate were censored, and entered as a "0," since death occurred for a nonrelated reason. Significance, as defined as a *P*-value < 0.05, was assessed using the Gehan-Breslow test. This test assumes that data from earlier survival times are more accurate than later times and weights these data accordingly. Data were combined from three plates, and another independent experiment gave the same results.

In vitro phenotypic assays

For in vitro assays, C. albicans strains were grown overnight at 30° in YPD, resuspended to OD = 1, serially diluted 1:5 in sterile water, and spotted on agar plates containing SDS, Calcofluor White, NaCl, Sorbitiol or paraquat at 0.04%, 20 µM, 0.5 M, 1.5 M, and 1 mM, respectively. For filamentation assays, strains were spotted on Spider media (1% nutrient broth, 1% D-mannitol, 2 g K₂HPO₄), and incubated at 37° for 7 days before being photographed. Ability to form biofilm was quantified using a previously described protocol (Reynolds and Fink 2001) with minor modifications. Briefly, C. albicans cells bound to polystyrene surfaces were stained with crystal violet, followed by washing to remove unbound dye and cells. Cells that remained bound after washing were quantified by measuring dye absorbance. Growth rate experiments were carried out using a Bio-Tek Synergy H4 microplate reader.

Ex vivo virulence assays

Two T75 flasks of RAW 264.7 mouse macrophage cells were grown to 80-90% confluence in DMEM + 10% FBS. Cells were scraped off the flask and 2 \times 10⁶ cells were plated in six-well plates (total volume 1 ml). Control wells of media only (DMEM + 10% FBS) were set up in parallel. RAW cells were allowed to adhere for 5 hr at 37°, 5% CO₂ and then 1.3×10^5 cells of an overnight *C. albicans* culture resuspended in DMEM + 10% FBS were spiked in each well at 1:15 multiplicities of infections (one yeast cell per 15 RAW 264.7 cells). Plates were incubated at 37°, 5% CO₂, overnight, scraped off each well, and resuspended in 10 ml 0.02% Triton-X 100 (v/v) to osmotically lyse the macrophages. The solution was then spun down, and the pellet resuspended in 1 ml sterile water. From 1:10 serial dilutions, 100 μ l of the 10⁴ and 10⁵ dilutions were plated on YPD. After incubating the plates overnight at 30°, colonies were counted, and percent killing determined by comparing cells counted in the plates with and without macrophages. Diphenyleneiodium chloride (DPI) experiments were performed as described above but with the addition of 0.05 μ M of DPI. Since the DPI was added from a stock concentration containing DMSO, control experiments without DPI were carried out with the same final concentration of DMSO.

Expression profiling

Overnight cultures of *C. albicans* were resuspended to $OD_{600} = 0.1$ in YPD, and allowed to grow for 4–6 hr to reach midexponential phase ($OD_{600} = 0.6$ –0.8). Cells were cold methanol quenched exactly as described in Thompson *et al.* (2013) right before the addition of 5 mM H₂O₂ or 5 and 15 min post H₂O₂ addition. Hydrogen peroxide is a suitable proxy for host response as it is the most common ROS used by the immune system during respiratory burst (Iles and Forman 2002), and passes through the cell membrane, reaching the cytoplasm quickly (Kohchi *et al.* 2009). We profiled

the expression of wild type, *zcf15/zcf15*, and *zcf29/zcf29* C. albicans, upon exposure to 5 mM H₂O₂ after 5 and 15 min, as the transcriptional response of C. albicans to H_2O_2 has been shown to peak at 10-15 min post H₂O₂ addition (Roy et al. 2013). The three time points we called, respectively, t_0 , t_5 , and t_{15} . Total RNA was extracted using the Qiagen RNeasy Plus Mini Kit according to the manufacturer's instructions. Briefly, 5 \times 10⁸ total cells were resuspended in 600 μ l buffer RLT with β -mercaptoethanol (10 μ l of β -mercaptoethanol for each 1 ml of RLT buffer), and transferred to 2 ml bead beating tubes; 500 µl RNAse-free Zirconia beads were added to the tubes, and the cells lysed by bead beating using a FastPrep instrument (MP Biomedicals). Tubes were centrifuged at 1000 \times g for 5 min at 4°, the supernatant transferred to a gDNA eliminator column, and RNA isolated following the manufacturer's instructions. RNA was quantified with a NanoDrop UV-Vis Spectrophotometer (Thermo Scientific), and with Agilent 2200 Tape Station.

From total RNA, mRNA was purified using Dynabeads mRNA DIRECT (Life Technologies 61006) according to manufacturer's instructions. mRNA was fragmented to 300-600 bp by adding 2 µl of zinc fragmentation buffer (Life Technologies AM8740) to 18 µl of mRNA, and heating the samples at 70° for 2 min. The fragmented mRNA was DNase treated using Turbo DNase enzyme (Life Technologies AM2238), and dephosphorylated using FastAP (Thermo Scientific EF0654). DNA was extracted and purified using Dynabeads MyOne Silane (Life Technologies 37002D), and a RiT-19 RNA adapter ligated at the 3' end of the mRNA. mRNA was reverse transcribed using AffinityScript RT enzyme using an rTd RT primer complementary to the RiT-19 RNA adapter. RNA was hydrolyzed with an alkali treatment, and cDNA purified using MyOne Silane beads. A 5iLL-22 adapter was added to the 5' end of the DNA, and cDNA libraries obtained with Phusion High-Fidelity DNA polymerase, and 10 total amplification cycles. Libraries fragment size and concentration were measured with the Agilent 2100 Bioanalyzer, and sequenced in a paired-end read format for 76 cycles on the Illumina HISeq platform.

RNA-Seq reads were aligned to the C. albicans SC5314 reference genome (version A21-s02-m08-r01, downloaded from http://www.candidagenome.org) using TopHat (Trapnell et al. 2012). For each strain, three biologically independent samples were sequences and aligned. Library quality was determined using RNA-SeQC (DeLuca et al. 2012) to measure the number of reads aligned, sequencing depth, and coverage (Figure S2, A-C, and Table S3). Gene expression was quantified using Cufflinks (Anders et al. 2012); to examine correlations, read counts were normalized by gene length and sample size, and expressed as reads per kilobases per million of reads (RPKM) as described here (Li and Dewey 2011). Statistically significantly differentially expressed genes (4-fold difference in RPKM, P-value cutoff for FDR < 0.001) were identified between conditions using edgeR (Robinson et al. 2010).

Quality control for RNA-Seq

We sequenced 153.7 million RNA-seq reads, of which 113.1 million reads mapped uniquely to the genome. The uniquely mapped rate was therefore \sim 74%, similar to previous studies (Tuch et al. 2010). For wild-type, zcf15/zcf15, and zcf29/zcf29, we obtained 49.3, 50.7, and 53.8 million total reads, respectively, of which 70, 76, and 75% uniquely aligned to the genome. The total number of reads was also evenly distributed across time points with 44.5, 66.6, and 42.6 million for samples extracted at T_0 (before the addition of H_2O_2), T_5 (5 min post addition of H_2O_2), and T_{15} (15 min after the addition of H₂O₂), respectively. Overall we obtained between 2 and 10 million reads per sample, and 70-80% of these reads uniquely aligned to the genome. This number of reads is adequate to draw meaningful conclusions in C. albicans, as it is estimated that high quality gene expression profiling can be achieved with 2 million reads per sample for yeast (Bailey et al. 2013). Reads aligned primarily to exonic regions (Figure S2A). A small number of reads (\sim 2–4%) aligned to intergenic region of the genome (possibly due to unannotated genes or sequencing error), and in a proportion similar to that found by others (Dhamgaye et al. 2012). In addition, very few reads aligned to intronic regions (\sim 3-4%), suggesting that our initial poly-A selection was effective in removing most of the genomic DNA, and that only mature mRNAs were sequenced in our samples.

We manually confirmed that *ZCF15* and *ZCF29* were not expressed from the respective knockouts. We used the Interactive Genomics Viewer (IGV) (Robinson *et al.* 2011) tool to visualize expression levels in the knockout loci (Figure S2B), and confirmed that no transcripts were detected from *zcf15/zcf15* and *zcf29/zcf29* strains, confirming that the genes were properly deleted.

Finally, we determined gene expression levels (RPKM) to evaluate gene expression reproducibility across biological replicates (Li and Dewey 2011). We made pairwise comparisons of \log_2 RPKM across each of our 27 samples. Sample correlation was measured using the Pearson correlation coefficient, which measures the strengths of a linear relationship between variables. We obtained a total of 729 (27 × 27) correlation coefficients, and summarized them as a heat map (Figure S2C). Biological replicates were highly correlated, demonstrating the reproducibility of the transcript counts. This high degree of transcriptional correlation between biological replicates was observed both before (Figure S2C, box 1), and after, the addition of H_2O_2 (Figure S2C, boxes 2 and 3).

Location profiling

To identify the transcriptional targets of Zcf15 and Zcf29, we used a Chromatin immunoprecipitation approach coupled with DNA Sequencing (ChIP-Seq). To selectively immunoprecipitate Zcf15 and Zcf29 protein-DNA complexes, we introduced the HA-epitope at their 3' so that their expression remained under the control of their endogenous regulatory

sequences (Figure S3). Proper genomic integration, expression, and function of the tagged proteins were confirmed by diagnostic PCR, Western Blot, and phenotypic characterization, respectively. In order to compare the ChIP-Seq results with the transcriptomic data, experiments were conducted under analogous conditions. Samples were split between "pulldowns" and "inputs", and, while "pulldowns" samples were immunoprecipitated using monoclonal anti-HA antibody, "inputs" were not, and were used as control samples. DNA-binding partners for each TF were analyzed at 5 and 15 min after exposure to 5 mM H₂O₂, and compared to untreated controls. Upon H₂O₂ exposure, samples were quickly cross-linked with formaldehyde, TF-bound DNA purified, and single-end libraries were constructed according to protocols provided by Illumina. Illumina sequencing yielded a total of 148 million 25-base sequence reads, with an average of 4.2 million reads per sample.

Reads were aligned to the C. albicans isolate SC5314 version A21-s02-m08-r01 genome using the Burrows-Wheeler Aligner (BWA) v0.7.4-r385 mem (Li and Durbin 2009) providing a mean coverage between $50 \times$ and $75 \times$. Alignments were converted to sorted BAM format using Samtools v0.1.9 (r783) (Li et al. 2009), and then converted to tagAlign format using BEDTools v2.17.0 (Quinlan and Hall 2010). Peaks were called according to the Irreproducibility Discovery Rate (IDR) framework using the peak caller MACS2 v2.1.0 (Zhang et al. 2008). Briefly, peaks for Zcf15 and Zcf29 were called on individual replicates using the untagged replicates as controls with relaxed thresholds ($n_{\text{peak}} = 300$ K). Peaks were next called on pooled replicates, and pseudoreplicates of individual and pooled replicates (shuffled and split into two files). Final peak calls were taken from merged replicates with a FDR threshold <0.05. SPP v1.10.1 (Kharchenko et al. 2008) was also assessed according to the IDR framework. The effective genome size for C. albicans was calculated using GEMTools (http://gemtools.github.io/) as 13,881,430 nt. Additionally, we used the strand crosscorrelation analysis of SPP to estimate the shift size parameter for each set of reads. Finally, we selected all peaks that were <300 nt upstream of a feature specified in the gene annotation file. Genome sequences spanning all IDR <0.05 ChIP-Seq peaks were subjected to de novo motif analysis using MEME-ChIP v4.10.11 (Machanick and Bailey 2011) against the JASPAR database (Mathelier et al. 2014), and including any number of motif repetitions, with a maximum width of 15 nt.

Gene set enrichment analysis (GSEA)

GSEA v2.2.1 was used to compare the differentially expressed genes from the RNA-Seq experiments of each zcf15 and zcf29 null mutant compared to their wild type. The numerically ranked \log_2 fold changes of each expression profile were compared to Candida Gene Ontology gene sets accessed from the Candida Genome Database (Inglis *et al.* 2012) using the "GseaPreranked" mode. Two output files reflecting enrichment for high or low fold change ratios for each GSEA analysis were used as input for the Cytoscape Enrichment Map app v2.0.1. The layout of the GO terms (nodes) was modified to the "edge-weighted spring embedded layout." The output of the Enrichment Map for *ZCF15* was a subset of the output for *ZCF29*, and was compared to *ZCF29* GO terms.

Data availability

The RNA-Seq and Chip-Seq data generated for this study are deposited in the GenBank SRA under Bioproject PRJNA356057.

Results

In vivo mutant screen to identify novel genes required for fungal virulence

We used a reverse genetic approach to identify genes required for virulence during infection of a live host by screening a collection of 724 C. albicans mutants (\sim 10% of the genome) (Table S2) using *C. elegans* as a model host (Jain *et al.* 2013). C. elegans is a useful model to study infectious disease. A rich body of literature demonstrates that molecular mechanisms of infectious disease progression in C. elegans are mechanistically similar to humans (Pukkila-Worley et al. 2009, 2011; also reviewed in Engelmann and Pujol 2010; Marsh and May 2012). We identified seven mutants, CMP1, IFF11, SAP8, DOT4, ZCF15, orf19.1219, and orf19.6713, representing 10% of the mutants screened, that were unable to illicit the Dar response, previously described as a robust disease phenotypes in C. elegans (Figure S4A), in particular, a deformity in the post anal region (Dar) (Jain et al. 2009). CMP1, IFF11, and SAP8 have previously been implicated in the virulence of C. albicans, thus validating our screening methods (Schaller et al. 1999a,b; Bader et al. 2003; Bates et al. 2007).

Mutations in the remaining four genes (DOT4, orf19.6713, orf19.1219, and ZCF15) unable to illicit the Dar response, are largely uncharacterized, and not previously linked to virulence (Figure S4B). Since the initial screen was conducted with transposon insertion mutants, we next tested homozygous deletion mutants available of three of the four novel mutants (Noble et al. 2010). Knockout mutants in DOT4, orf19.1219, and ZCF15 showed reduced ability to elicit signs of early infection to the same extent as the transposon insertion mutants. These results confirm that the phenotypes observed are likely due to the loss of function of these specific genes. DOT4 and orf19.1219 are predicted to be involved in ubiquitin metabolism and have human homologs (Figure S4B). In contrast, ZCF15 does not have a human homolog. Furthermore, ZCF15 belongs to a family of transcription factors that are expanded in pathogenic fungi, and absent in nonpathogenic fungi such as Saccharomyces cerevisiae (Figure 1A, Butler et al. 2009). ZCF15 is specifically expanded in pathogenic species with three paralogs in C. albicans, the highest count of any ZCF family, making it a good candidate for further study.

The zcf15/zcf15 and zcf29/zcf29 null mutants are sensitive to ROS

To characterize the mechanism of the reduced virulence of *zcf15/zcf15*, and other Zcf family mutants, we phenotypically profiled the 10 Zcf transcription factors that are conserved in pathogenic fungi, but absent in nonpathogens. Mutants were tested in *in vitro* assays, including biofilm formation, dimorphic transition from yeast to hyphal morphology, and exposure to various stressors including temperature, osmotic, alternate carbon sources, nitrogen starvation, and other drugs (Mitchell 1998; Ramirez and Lorenz 2007; Heller and Tudzynski 2011; Wachtler et al. 2011). The zcf15/zcf15 and zcf29/zcf29 mutants were sensitive to oxidative stress conditions (Figure 2A; and Castell et al. 2005). ZCF29 is upregulated in the presence of H₂O₂ (Enjalbert et al. 2006), and zcf29/zcf29 mutant is sensitive to the ROS mimic menadione (Homann et al. 2009). ZCF29 was not represented in the in vivo library screen where ZCF15 was identified. We therefore obtained the null mutants from the FGSC (Table S1) for this study.

The ROS sensitivity phenotype of zcf15/zcf15 null mutants was complemented by the reintroduction of a single copy of the $zcf15/ZCF15^{C}$ gene in either the endogenous locus or at an ectopic location. The sensitivity profile of the complemented strain was indistinguishable from the wild type (Figure S5), suggesting that *in vitro* hypersensitivity of zcf15/zcf15 to ROS was a result of the intended deletion of ZCF15. We were unable to generate a complemented version of the zcf29/zcf29 null mutant using multiple methods and markers. The zcf29/zcf29 null mutant used in this study has been previously verified (Homann *et al.* 2009), and we tested two independent isolates of the zcf29/zcf29 null mutant to confirm its phenotype. Together, these studies suggest that ZCF15 and ZCF29 confer resistance to ROS, a key component of the innate host response to *C. albicans* infection.

Zcf15 and Zcf29 are required to withstand host generated ROS and establish a sustained infection in vivo

To test if the reduced virulence of *zcf15/zcf15* and *zcf29/zcf29* was due to the inability to withstand host-generated ROS, we tested the ability of these mutants to establish an infection in ROSdeficient bli-3 mutant worms. The bli-3 gene encodes the dual oxidase (CeDuox1) that is involved in creating an environment of elevated ROS. We, and others, have previously demonstrated that the lifespan of the bli-3 mutant is indistinguishable from the wildtype when grown on Escherichia coli OP50, suggesting that these mutants are otherwise healthy (Chavez et al. 2009; Jain et al. 2009). The average lifespan of C. elegans infected with wild-type C. albicans was significantly shorter than those infected with the zcf15/zcf15 and zcf29/zcf29 mutants (Figure 2B). Strikingly, the survival plots of ROS-deficient bli-3 mutant C. elegans infected with C. albicans are indistinguishable between the zcf15/zcf15 and zcf29/zcf29 mutant, and wild type (Figure 2C). Together these data indicate that both ZCF15 and ZCF29 are required to mitigate the effects of host-derived ROS, since these gene products are not required in a host that is unable to produce ROS.



Figure 2 The *zcf15/zcf15* and *zcf29/zcf29* null mutants are sensitive to oxidative stress. (A) The deletion mutants of *zcf15/zcf15* and *zcf29/zcf29* are sensitive to 1 mM paraquat and 80 μ M menadione, respectively. They are sensitive to *in vitro* oxidative stress conditions as compared to the wild type controls. Survival curves for (B) wild type N2 worms exposed to *zcf15/zcf15* and *zcf29/zcf29* mutants survive longer than worms exposed to wild-type *C*. *albicans*, while (C) *bli-3* mutant worms, which lack the ability to produce ROS, exposed to *zcf15/zcf15* and *zcf29/zcf29* null mutants, are indistinguishable from those infected with wild-type *C*. *albicans*. (D) Wild-type *C*. *albicans* survive exposure to cultured macrophages significantly better (P < 0.01) than the *zcf15/zcf15* and *zcf29/zcf29* mutants (light gray bars). Error bars indicate SE. Macrophages treated with DPI, an agent that inhibits the production of ROS by inhibiting the activity of the NADPH oxidase (dark gray bars), exhibit wild type levels of survival. Since the DPI was added from a stock concentration containing DMSO, control experiments without DPI were carried out with the same final concentration of DMSO. Experiments were performed in triplicate, biologically replicated to ensure reproducibility of results presented.

Next, we wanted to test the role of Zcf15 and Zcf29 in neutralizing ROS produced by phagocytes. Phagocytes rely on the oxidative burst as their primary defense mechanism against *C. albicans* (Newman and Holly 2001; Ashman *et al.* 2004; VanderVen *et al.* 2009). Wild-type *C. albicans* can effectively neutralize ROS to survive (Wysong *et al.* 1998; Lorenz *et al.* 2004; Arana *et al.* 2007; Frohner *et al.* 2009; Wellington *et al.* 2009), and eventually cause macrophages to lyse. To test if Zcf15 and Zcf29 were required for neutralizing ROS within a phagosome, we measured the relative sensitivity of macrophages

ex vivo exposed to wild type *C. albicans* compared to the *Zcf* null mutants (*zcf15/zcf15* or *zcf29/zcf29*). *zcf15/zcf15* were significantly more susceptible to macrophage killing compared to wild type (P < 0.023, Figure 2D). This effect was eliminated in the presence of DPI, an inhibitor of the NADPH oxidase that has been shown to decrease fungicidal activity against *C. albicans* by decreasing ROS production within macrophages (Donini *et al.* 2007). These results suggest that one mechanism of *in vivo* susceptibility is that Zcf15 and Zcf29 are required to neutralize the oxidative environment within the phagosome.

Zcf15 and Zcf29 function in an interconnected transcriptional network regulating macro and micronutrient homeostasis

To understand the role of Zcf15 and Zcf29 in mediating resistance to ROS and virulence, we profiled gene expression of each transcription factor using RNA-Seq in the presence, and in the absence, of hydrogen peroxide (H_2O_2). Transcriptional profiles obtained for each strain before and after addition of H_2O_2 were dramatically different, as suggested by their low degree of correlation (Figure S2C, dashed blue rectangle). This indicates that H_2O_2 triggers a large transcriptional response within 5 min (T_5) that is even more dramatic after 15 min (T_{15}) as compared to control time point (T_0). Furthermore, biological replicate samples at T_5 were highly correlated with each other, but poorly correlated with those at both T_0 and T_{15} , suggesting that the T_5 samples effectively captured an intermediate step in the H_2O_2 driven transcriptional rewiring obtained between T_0 and T_{15} .

RNA-Seq for both of the *ZCF* knockouts indicated that *ZCF29* regulates a larger set of genes (Figure 3A). Major cellular processes including respiration, amino acid metabolism, ribosome assembly, and proteasome functions are dysregulated in the null mutants (Figure 3A). Deletion of *ZCF29* has a greater overall effect on expression levels compared to the deletion of *ZCF15*; 1402 genes were differentially expressed in *zcf29/zcf29*, 168 genes differentially expressed in *zcf15/zcf15*, and the average log expression increased by 0.25 in *zcf29* compared to 0.05 for *zcf15*. This suggests that both genes function as transcriptional repressors, with a larger set of genes regulated by *ZCF29*. In the absence of *ZCF29*, additional *ZCFs* are significantly upregulated, including *ZCF1*, *ZCF10*, *ZCF24*, *ZCF39*, and *ZCF9*, indicating it is a master regulator of *ZCFs*, while *zcf15/zcf15* was not found to change regulation for additional *ZCFs*.

In the absence of H₂O₂, ZCF15 and ZCF29 regulate genes involved in carbohydrate and nitrogen metabolism, respectively, two macronutrients that are critical determinants of overall fitness of C. albicans during the pathogenic state. Strikingly, 105 of the 107 genes coregulated between the zcf29/zcf29 and *zcf15/zcf15* mutants have the same directionality of expression (Figure 3B). These 105 coregulated genes include 50 upregulated and 55 downregulated genes (Figure 3B). GSEA revealed that genes required to maintain homeostasis of transition metals such as iron are enriched in this list of coregulated genes. For example, IRO1 is downregulated (1.99-fold and 2.07-fold in zcf15/zcf15 and zcf29/zcf29 respectively). Iro1 is a putative transcription factor that has been shown to play a role in iron utilization. On the other hand, Hap43, a repressor that mediates responses to low iron, and Hmx1, a heme oxygenase that is required for iron utilization, are both upregulated (HAP43 1.65-fold and 1.70-fold in zcf15/zcf15 and zcf29/zcf29, HMX1 1.67-fold and 1.97-fold in zcf15/zcf15 and zcf29/zcf29). This expression pattern is consistent with that observed when iron availability is low (Lan et al. 2004).

The availability of iron in cells is tightly regulated because it serves as an electron donor or acceptor in the formation of toxic free radicals. For example, the oxidation of Fe(II) to Fe(III) is required for the conversion of H_2O_2 to the more toxic hydroxyl radical OH• ROS (Winterbourn 1995). Within host niches, which differ markedly in the levels of bioavailable iron, the ability of *C. albicans* to regulate iron homeostasis has been shown to be a critical aspect of the pathogenic lifestyle (Noble 2013). We speculate that the misregulation of genes involved in iron homoeostasis alters the availability of intracellular free iron, which results in the hypersensitivity of *zcf29/zcf29* and *zcf15/zcf15* to ROS.

Zcf29 regulates amino acid bioavailability

Genes involved in amino acids biosynthesis as well as proteolysis are dysregulated in zcf29/zcf29 but not zcf15/zcf15 (Figure 3C). Multiple amino acid biosynthetic pathway genes including threonine, methionine, lysine, histidine, serine, leucine, tyrosine, and phenylalanine, are all downregulated in the *zcf29/zcf29* null mutant (Figure 3C). Five of the six genes involved in methionine biosynthesis were significantly downregulated (HOM3 2.1-fold, HOM2 1.8-fold, HOM6 1.4fold, MET2 1.8-fold and MET6 1.6-fold), while four of the five genes involved in threonine biosynthesis were significantly downregulated (HOM3 2.onefold, HOM2-1.8 fold, HOM6 1. fourfold, THR4 1.4-fold). In contrast to amino acid biosynthesis, loss of function of ZCF29 leads to upregulation of genes involved in protein degradation, both ubiquitin dependent and independent pathways. The increased protein turnover in the zcf29/zcf29 mutant may be an effect of decreased bioavailability of amino acids, since, with the exception of arginine biosynthesis, all amino acid biosynthesis is downregulated in the zcf29/zcf29 mutant. Alternatively, it may be a consequence of the protein damage caused by ROS.

In contrast to methionine and threonine biosynthesis, genes involved in arginine biosynthesis are upregulated up to 23-fold in zcf29/zcf29 mutants. Six of the seven genes involved in arginine biosynthesis are upregulated in zcf29/ zcf29 (ARG1 23-fold, ARG3 19-fold, CPA1 12-fold, ARG8 11-fold, CPA2 eightfold, and ARG5, 6 sixfold). Interestingly, the only gene in the pathway not induced in our data was ARG2, which is largely regulated post-translationally in S. cerevisiae (Wipe and Leisinger 1979). This induction is not driven by an arginine deficiency, because cells were grown in rich media, and isogenic wild type and zcf15/zcf15 mutants do not exhibit this profile. The arginine biosynthetic pathway has been linked to ROS resistance. Interestingly, C. albicans upregulates the arginine biosynthetic pathway, expect ARG2, upon exposure to macrophage-generated ROS (Jimenez-Lopez et al. 2013). C. albicans cells phagocytosed by macrophages that cannot produce ROS due to a deficiency in the gp91 (phox) subunit of the oxidase do not upregulate this pathway, corroborating our findings linking arginine bioavailability and resistance to host-derived ROS.

Zcf15 controls carbon metabolism and cellular energy production

Deletion of zcf15 results in misregulation of genes involved in carbohydrate metabolism. Carbohydrates (Figure 3D) are the



Figure 3 Differential expression analysis highlights modules important for response to oxidative stress coordinated by Zcf15 and Zcf29. (A) Differentially expressed gene networks. In the zcf29/zcf29 and zcf15/zcf15 mutants 1402 and 168 genes, respectively, were differentially regulated in the mutant relative to the wild type control. Together, this represents 25% of the C. albicans genome. The cyan and yellow balls represent GO term enrichment in the zcf15/zcf15 or zcf29/zcf29 mutant, respectively. The color of the border represents opposite ends of the expression changes: green highlights downregulated gene sets, red highlights upregulated gene sets. The size of each GO term reflects the number of genes assigned to that term, and the width of the lines reflects the "overlap coefficient" ([size of (A intersect B)]/{size of [minimum (A, B)]}) of the GO terms they connect. (B) A total of 107 genes are dysregulated in both zcf29/zcf29 and zcf15/zcf15 mutants. Genes required to maintain homeostasis of transition metals such as iron are enriched in this list of coregulated genes. (C) The biosynthesis of the amino acid threonine (light blue), methionine (green), lysine, histidine, serine, leucine, tyrosine, and phenylalanine (purple), are downregulated the zcf29/zcf29 null mutant. Genes involved in various aspects of proteolysis are upregulated in the same mutant. Specifically, 18 genes involved in ubiquitin mediated proteolysis, two ubiquitin ligase genes, six genes involved in proteasome assembly, six genes directly involved in proteasome mediated degradation (amino and carboxyl peptidases), one gene involved in ubiquitin recycling (marked in brown). Differential gene expression was reported as log₂FC on the X-axis and statistical significance [–log10 (P value)] on the Yaxis. Genes statistically significantly upregulated in the zcf29/zcf29 mutant compared to wild type are reported as red dots on the right side of the graph, while genes statistically significantly downregulated as red dots to the left of the graph. Genes not statistically significantly differentially expressed are reported as black dots. (D) Genes involved in glycolysis, carbon metabolism, and ATP production are downregulated in the zcf15/zcf15 null mutant. Specifically, the glucose transporters SHA3, HGT7, and HGT8; Tye7, a transcription factor controlling glycolysis; Pck1 the PEP carboxykinase, Pyc2 a Pyruvate carboxylase, Mae1 a malate dehydrogenase, Nde1 a NADH dehydrogenase, and Stf2 a protein involved in ATP biosynthesis, are all significantly downregulated. Differential gene expression was reported as log₂ fold change on the X-axis and statistical significance [-log10 (P value)] on the Y-axis. Genes statistically significantly upregulated in the zcf15/zcf15 mutant compared to wild type are reported as red dots on the right side of the graph, while genes statistically significantly downregulated as red dots to the left of the graph. Genes not statistically significantly differentially expressed are reported as black dots.

preferred source of energy for *C. albicans*, are metabolized during glycolysis and the citrate cycle, and used for the biosynthesis of essential components, including amino acids. The 95 genes significantly downregulated in *zcf15/zcf15* have enriched biological functions related to carbon utilization (P < 0.027); including *CAT2*, *MIG1*, *MAE1*, *PYC2*, and *PCK1*, which are all downregulated between two and fivefold (Figure 3B). *CAT2* is a major carnitine acetyl transferase involved in the transport of acetyl-CoA produced during peroxisomal fatty acid β -oxidation to the mitochondria, where it can enter the TCA cycle, and can be oxidized completely to CO_2 and H_2O (Strijbis *et al.* 2008). *MIG1* is a transcription repressor that regulates carbon utilization (Zaragoza *et al.* 2000); the *S. cerevisiae MIG1* is a TF that shuttles between the cytosol and nucleus depending on external glucose levels (Schuller 2003). *MAE1*, *PYC2*, and *PCK1* catalyze the first three steps of gluconeogenesis: the oxidation of malate to pyruvate, the subsequent carboxylation of pyruvate to oxaloacetate, and the final conversion of oxaloacetate to phosphoenolpyruvate. Thus, the Zcf15-mediated response is reflected in the expression levels of genes encoding enzymes that modulate fungal growth. Growth of *C. albicans in vitro* or within a live host is dependent on nutrients and the availability of energy sources.

Together, Zcf15 and Zcf29 control \sim 25% of the *C. albicans* transcriptome encompassing the basic nutritional mechanisms of the cell. These two transcription factors control the metabolism of nitrogen and carbon, key nutritional cues for the regulation of virulence in *C. albicans*.

Zcf29 and Zcf15 regulate oxidative stress responses via noncanonical pathways

A striking phenotype of zcf15/zcf15 or zcf29/zcf29 null mutants is their avirulence in nematodes, except in *bli-3* mutants, which do not produce ROS (Figure 2C). To further characterize how Zcf15 and Zcf29 mediate the transcriptional response to ROS, we analyzed the RNA-Seq experiment and determined which genes were significantly differentially expressed between wild type and zcf15/zcf15 and zcf29/zcf29 mutants, in the presence or absence of hydrogen peroxide at 5 or 10 min, postexposure. We compared the transcriptional response triggered by sublethal concentration (5 mM) of H₂O₂. H₂O₂-responsive genes were identified that were regulated directly or indirectly by Zcf15 (210 genes) and Zcf29 (668 genes), respectively.

To test whether the zcf15/zcf15 and zcf29/zcf29 mutants are experiencing global redox homeostasis defects in the absence of oxidative stress, we evaluated the regulation of key genes in four major pathways that regulate response to oxidative stress in *C. albicans*. Specifically, we tested the superoxide pathway, the thioredoxins pathway, the glutathione pathway, and *CAP1* pathway. Our results indicate that key genes in these pathways are not misregulated (Figure S6A), suggesting that the zcf15/zcf15 and zcf29/zcf29 mutants are not experiencing global redox defects. Furthermore, genes in these pathways are upregulated to an extent that has been previously demonstrated (Wang *et al.* 2006). Together, these results suggest that zcf15/zcf15 and zcf29/zcf29 mutants are not experiencing global redox stress, and likely regulate ROS detoxification via noncanonical mechanisms.

To analyze the hydrogen peroxide responsive genes, we clustered them into four groups based on their transcriptional responses (Figure S6, B and C) (Robinson *et al.* 2010). Groups 1 and 2 contain genes that are less (group 1) or more (group 2) downregulated by H_2O_2 in the mutant compared to the wild type. Groups 3 and 4 contain genes that are less

(group 3) or more (group 4) upregulated by H_2O_2 treatment in the mutant compared to wild type.

To identify the biological functions enriched between wild type and zcf15/zcf15 upon H₂O₂ challenge, we used GO term analysis for groups 1 and 4 (Inglis et al. 2012). Genes involved in carbon utilization ($P = 2.5 \times 10^{-5}$) and oxidation-reduction processes ($P = 1 \times 10^{-4}$) were enriched (Figure 4A). These two biological processes are highly interconnected, as it has been shown that, in the presence of ROS, C. albicans presents a "starvation like" phenotype by downregulating genes involved in carbon metabolism, and simultaneously upregulating genes involved in oxidation reduction processes and ROS detoxification (Lorenz et al. 2004). Genes involved in oxidation reduction processes/ROS detoxification including DOT5 (a thiol peroxidase), IFD6 (a aldo-keto reductase), OFD1 (prolyl hydroxylase), and AMO1 (a peroxisomal oxidase), are upregulated in *zcf15/zcf15* to a statistically significantly yet lower extent. These genes likely represent potential targets of Zcf15 mediated repression. The reduced ability of *zcf15/zcf15* to upregulate these genes in the presence of H₂O₂ may explain the hypersensitivity to ROS.

In the *zcf29/zcf29* mutant, 110 genes were downregulated by H₂O₂ compared to wild type. These genes (group 1, Figure S6C) are potential targets of *ZCF29* repression, as their full downregulation depends on *ZCF29*. GO term analysis revealed that genes involved in ribosome assembly are overrepresented in group 1 (corrected *P* value 1.4×10^{-18}) suggesting that, in the absence of *ZCF29*, many of these genes are misregulated, as they are not fully downregulated upon exposure to H₂O₂ (Figure 4B).

Under ROS stress, C. albicans downregulates ribosome biogenesis in order to liberate energy resources for other cellular processes (Loar et al. 2004) since ribosome biogenesis and assembly is energetically demanding. Our data suggests that the ability to downregulate ribosome biogenesis upon ROS challenge is compromised in zcf29/zcf29 as many of the genes involved in the various steps of ribosome biogenesis are downregulated to a significant lower degree in zcf29/zcf29. For example, UTP21, NAN1, UTP5, UTP20, and UTP13 are five U3 snoRNA proteins involved in pre-rRNA processing. DBP2 and DBP3 are two DEAD-box RNA helicases involved in rRNA maturation, RCL1 is an endonuclease involved in 90S preribosome processing, and REI1 is a cytoplasmic pre-60S subunit protein, all of which are downregulated to a lower degree in zcf29/zcf29 compared to wild type. Many of these genes have been shown to be downregulated upon macrophage generated ROS (Lorenz et al. 2004), suggesting that these genes are downregulated not only in the presence of ROS in vitro, but also in the context of a mammalian infection.

We also identified specific genes that are dysregulated in the presence of oxidative stress, which may account for the ROS sensitivity phenotype of *zcf15* and *zcf29* mutants. For example, upon H_2O_2 treatment, *SOD1*, the superoxide dismutase that protects *C. albicans* from oxidative stress, is



Figure 4 Under oxidative stress conditions Zcf15 and Zcf29 are required to downregulate energy expensive functions. (A) ZCF15 is required for the upregulation of genes required for ROS detoxification (*P*-value = 1×10^{-4}), and the downregulation of carbon metabolism (*P*-value = 2.5×10^{-5}). (B) ZCF29 is required for downregulation of ribosome biogenesis (*P*-value 1.38×10^{-18}) when exposed to H₂O₂.

downregulated 2.45-fold in *zcf29/zcf29*, and *GAL10* is downregulated 4.3-fold in *zcf15/zcf15* as compared to wild type; GAL10 encodes a UDP-glucose 4 epimerase, and loss of its function results not only in its inability to grow on galactose as the sole carbon source, but also to an increased H_2O_2 susceptibility, suggesting that the function of this gene is not limited to the catabolism of exogenous galactose (Singh et al. 2007). Another gene involved in ROS response signaling, CMK1, is 2.1-fold downregulated in zcf15/zcf15 compared to wild type upon H_2O_2 treatment. CMK1 codes for a calcium/calmodulin-dependent protein kinase that has a role in both cell wall architecture and oxidative response (Ding et al. 2014); cmk1 null mutants showed an increased intracellular ROS levels compared to wild type when exposed to H_2O_2 , suggesting that this gene plays an important role in ROS detoxification. The dysregulation of these genes therefore supports a role for ZCF15 and ZCF29 in the transcriptional response to ROS.

Taken together, our data suggest that the downregulation of ribosome biogenesis under ROS stress is compromised in *zcf29/zcf29*. The *C. albicans* ability to downregulate this very energy demanding process upon ROS exposure is critical, and we believe that the *zcf29/zcf29* inability to do so may be the underlying cause of its hyper-susceptibility to ROS.

Zcf15 and Zcf29 relocate to different genomic loci upon H₂O₂ exposure

Epitope-tagged Zcf15 and Zcf29 were generated and verified (Figure S6). Chromatin immunoprecipitation coupled with DNA sequencing (ChIP-Seq) was used to identify the genomic sites bound by Zcf15 and Zcf29 (Nobile *et al.* 2009) (*Materials and Methods*) (Figure S7 and Table S4). Samples from 5 and 15 min after the addition of 5 mM H₂O₂ were compared to unexposed samples (T_0). In the absence of H₂ O₂, we identified 151 and 141 regions bound by Zcf15 and

Zcf29, respectively. In the presence of H_2O_2 , we identified 93 and 210 regions bound by Zcf15 and Zcf29, respectively (IDR <0.05). By focusing on regions that were \leq 300 bp upstream of annotated genes, we narrowed our list to a high confidence set of 32 and 84 candidate genes directly bound by Zcf15 and Zcf29, respectively, six of which were bound by both TFs (Figure 5A). The subset of these candidate regulated genes found in the presence or absence of H₂O₂ differed greatly, as only 4 Zcf15 and 41 Zcf29 targets were detected in both conditions, suggesting that Zcf15 and Zcf29 relocate to different genomic loci upon H₂O₂ exposure, confirming our transcriptome analysis that showed regulations of different sets of genes in the presence or absence of H_2O_2 . Orthology to S. cerevisiae revealed that three of these conserved targets, as well as nine targets bound by one ZCF, form an interconnected network, which includes proteins with diverse function (Figure 5B). The genes in this connected module include two mitochondrial proteins, Mim1 and Fmp10. Three of the six genes targeted by both ZCFs are part of this module.

In the absence of H_2O_2 , four of the 18 Zcf15 peaks overlapped Zcf29 peaks. Orthologs of these four genes have diverse functional roles. They include ascospore wall assembly (*SPO75*), meiotic DNA recombination (*MEI5*), and vesicleassociated transport (*PEP12*). The remaining target, *IFD6*, encodes an aldo-keto reductase, and is involved in biofilm formation; this gene is significantly upregulated in both *zcf15/zcf15* and *zcf29/zcf29*, suggesting both genes repress *IFD6* expression in the absence of stress exposure. This gene had a single upstream peak at T₀ for Zcf15, and two distinct peaks upstream for Zcf29—one that was present at T₀ and T₅, and the other that was present at all three time points (T₀, T₅, and T₁₅). These findings suggest that Zcf binding at multiple upstream sites differentially represses this gene.

Zcf15 differs from Zcf29 in that it binds to a higher fraction of sites in the absence of H_2O_2 . Over half of the peaks for Zcf15



Figure 5 Genes <300 bp upstream of high confidence *ZCF15* and *ZCF29* Chip-Seq peaks. (A) Venn diagram of counts of genes linked to ZCF peaks in the presence and absence of H_2O_2 . (B). Genes <300 bp upstream of peaks for *ZCF15* (blue), *ZCF29* (red), or both ZCFs (purple), are depicted. Genes highlighted in yellow were also identified during transcriptomic analysis. Using orthology to *S. cerevisiae*, network connections were inferred, and an interconnected module identified that includes three of the six genes bound by both ZCFs.

(18 of 32), and roughly a quarter of the peaks for Zcf29 (19 of 84), were found only at T₀; binding was not detected at these locations following H₂O₂ exposure. Genes uniquely directly regulated by Zcf15 include ribosomal proteins RPL5 and 60S ribosomal protein L7, a glucosyltransferase involved in cell wall mannan biosynthesis (*ALG8*), and an uncharacterized gene (*ORF19.7013*) that is upregulated 4.7-fold upon H₂O₂ exposure. Many of the peaks at T₅ and T₁₅ for Zcf15 were closest to genes for tRNAs (n = 5) or snoRNA (n = 4), three of which were identified at T₀ and T₅, and one at only T₅ and T₁₅.

ChIP-seq revealed a larger number of Zcf29 peaks than Zcf15 peaks, which correlates with the larger impact of *zcf29* deletion on expression. Upon exposure to H_2O_2 , genes involved in the heat shock response (*HSP70*), and several transporters (*CDR1*, *CDR4*, *FLU1*, and *HGT7*), were bound at T₅; transcription *HSP70* is upregulated at this time point, while the *MFS* transporter Hgt7 is significantly repressed. Notably, after 15 min of H_2O_2 exposure, Zcf29 binds specifically near five genes, of which four are known to be involved in the response to oxidative stress. These include *CIP1* (oxidoreductase), *CCP1* (cytochrome-*c* peroxidase), *EBP1* (NADPH oxidoreductase), and *HYR1* (glutathione peroxidase). This

highlights the specificity of Zcf29 for binding to genes involved in oxidative stress after 15 min of H_2O_2 exposure.

We performed *de novo* motif discovery and enrichment from the ChIP-Seq peaks, identifying distinct motifs for Zcf15 and Zcf29 bound sites (Figure S8). For each Zcf, multiple unrelated motifs were found to be significantly enriched. This result is consistent with the observation that there is a gross change in the ZCF-bound region before and after exposure to H_2O_2 . A comparison to known motifs found similarity between an adenine-rich motif for Zcf29 and the binding site for Sfl1, which activates stress response pathways and represses flocculation (Bauer and Wendland 2007). The similar significance values for multiple motifs suggest there may not be a preference for a highly conserved binding single site for both ZCFs under the conditions tested.

To summarize the significant findings of the genomic-scale RNA-Seq and ChIP-Seq analysis: 23% of the Candida transcriptome is misregulated in the absence of *ZCF15* and *ZCF29*. The genes regulate metabolic processes of macro (carbon and nitrogen) and micronutrient (iron) control in the absence of oxidative stress, which likely contributes to pathogen fitness, especially within the host niches. Under oxidative stress, Zcf29 downregulates ribosome biogenesis, and genes involved in ROS detoxification are misregulated in the *zcf15* null mutant. Comparison of our datasets with previous published work (Wang *et al.* 2006) suggests that ROS resistance is mediated via novel pathways that bypass the well-characterized Hog1 and Cap1 pathways, since genes involved these classical pathways were not misregulated in these mutants. Comparison of ChIP-Seq targets in the presence and absence of H_2O_2 indicates that Zcf15 and Zcf29 relocate to different genomic targets, which is consistent with identification of multiple biding motifs. This evidence confirms and validates our RNA-Seq experiments showing that Zcf15 and Zcf29 control different biological functions in the presence and absence of H_2O_2 .

Discussion

Integrating metabolic inputs is crucial to *C. albicans* pathogenicity. In addition to the obvious platform for nutrient assimilation and growth in diverse host niches, metabolism also supports other less obvious fitness attributes such as antioxidant production, protein turnover, macromolecule repair. and generating precursors for energy required for the cell. Metabolism also contributes to virulence by enhancing stress adaptation (Brown *et al.* 2014). For example, carbon adaptation is important for cell wall architecture. and also modulates immune surveillance (Ene *et al.* 2012a,b).

Here, we describe a complex interconnected regulatory circuit driven by two fungal specific TFs that link the impact of metabolic and stress adaptations to virulence and immunogenicity. The ZCF class of TFs is expanded within the lineage of pathogenic Candida (Figure 1, A-C); however, their specific functional roles are largely not yet understood. In vitro phenotypic characterization revealed that ZCF15 and ZCF29 are required to respond to ROS, a critical aspect of host innate immune response. Consistent with this finding, the mutants are less virulent in C. elegans as well as in cultured macrophages, where innate immunity plays a central role in resistance to C. albicans infections (Figure 2B). Furthermore, infection of the C. elegans bli-3 mutant unable to produce ROS is susceptible to infection by zcf15/zcf15 and zcf29/ zcf29 mutants (Figure 2C). The zcf15/zcf15 and the zcf29/ zcf29 mutants are both able to survive cultured macrophages whose ability to generate ROS has been pharmacologically inhibited, but not in those that can generate ROS, further confirming that these genes are required to protect the pathogen against innate host defenses (Figure 2D).

Our analysis indicates that, with the exception of arginine biosynthesis, *de novo* synthesis of amino acids is repressed in Zcf29. We hypothesize that the upregulation of arginine, a precursor of reactive nitrogen species (RNS) may help *C. albicans* resist the host innate immune defenses via production of RNS. In addition to ROS production, phagosomes produce RNS as an innate immune response. RNS are produced by nitric oxide synthase, an enzyme that converts arginine to citrulline with simultaneous production of nitric oxide (Marletta *et al.* 1988). Nitric oxide is subsequently converted

to more toxic RNS, like nitrogen dioxide radicals (NO₂) and peroxynitrite (ONOO⁻) that have a fungicidal effect on *C. albicans*. The *zcf29/zcf29* mutant, which is unable to resist host derived ROS, upregulates arginine biosynthesis. This shifts the equilibrium such that increased bioavailability of arginine within that phagosome decreases RNS production. Although this hypothesis requires further biological validations, our data reinforce the poorly understood connection between arginine reserves and *C. albicans* virulence that has been recently reported by others (Jimenez-Lopez *et al.* 2013).

Genomic analysis revealed a large network that plays a critical role in orchestrating nutritional needs. Zcf15 and Zcf29 regulate carbon and nitrogen metabolism, respectively, two major nutritional requirements that contribute to pathogen fitness (Madhani and Fink 1998; Lorenz and Fink 2002; Brown et al. 2014). Together they regulate iron metabolism, another key micronutrient required for pathogen fitness during infection. Nutrient availability contributes directly to pathogen growth and reproduction, therefore defining its success as a pathogen. Our data suggests that dysregulation of the biochemical nutritional pathways in zcf15/zcf15 and *zcf29/zcf29* mutants results in their inability to respond to host-related stresses, and, thereby, reduced virulence. The ability of C. albicans to rapidly and dynamically respond to changes in the host microenvironment is compromised if the function of Zcf15 or Zcf29 is impaired.

In the ecological niche of a host, the ability of a pathogen to reproduce sooner, faster, or in higher numbers, increases its fitness capacity, enabling it to perhaps live longer, survive against antimicrobial therapies, or disseminate and infiltrate deeper tissues and organs (Brown et al. 2014). As a commensalpathogen, C. albicans coevolves with its human host, where these traits likely arise due to mutations. In particular, the ZCF family appears well suited to give rise to adaptive mutation due to recent expansion of this family in the pathogenic Candida species. The role of ZCF15 and ZCF29 in oxidative stress appears newly acquired, based on the lack of conservation outside pathogenic fungi, and lack of similarity to conserved transcription factors known to regulate oxidative stress, such as CAP1 (Mogavero et al. 2011; Schubert et al. 2011; Jain et al. 2013). Further study of additional related Zcf proteins may help to uncover how these novel regulatory patterns evolved.

Both ZCF circuits appear to be based largely, if not exclusively, on negative regulation. Zcf15 and Zcf29 relocate to different genomic loci upon H_2O_2 exposure. Zcf29 plays a critical role in the H_2O_2 -dependent upregulation of a predicted aldo-keto reductase (*IFD6*). While not previously shown to be involved in the oxidative stress response in *C. albicans*, *S. cerevisiae* strains aldo-keto reductase genes appear to have lost regulation of oxidative stress markers (Chang and Petrash 2008). *IFD6* is conserved in *C. dubliniensis* and *C. tropicalis*, but does not have clear orthologs in more distantly related species, as the aldo-keto reductase family includes multiple genes in *Candida* species. Overall, our data sheds light on the genes and biological functions



Figure 6 Summary of cellular processes regulated by ZCF15 and ZCF29. Together, these genes regulate the response to nutritional cues during pathogenic interactions including iron, carbon, and nitrogen metabolism.

controlled by Zcf15 and Zcf29, which play a critical role in resistance to ROS in *C. albicans*.

Together, Zcf15 and Zcf29 regulate the expression of nearly a quarter of the genome. Such large circuits have previously been shown to regulate key biological processes such as biofilm formation in C. albicans (Nobile et al. 2012), the control of osmotic stress and pseudohyphal growth pathways of S. cerevisiae (Borneman et al. 2006; Ni et al. 2009), competence and spore formation in Bacillus subtilis (Losick and Stragier 1992; Suel et al. 2006; de Hoon et al. 2010), the hematopoietic and embryonic stem cell differentiation pathways of mammals (Wilson et al. 2010; Young 2011), and the regulation of circadian clock rhythms in Arabidopsis thaliana (Alabadi et al. 2001; Locke et al. 2005), each of which orchestrate a large set of target genes. One consideration for maintaining such a large circuit is its integration into a wide range of nutritional and environmental cues to produce an appropriate functional output under different conditions (Figure 6). An alternate hypothesis is the large structure of the network represents an overlapping regulon with feedback loops to ensure a coordinated cooperation during infection. Yet another possibility is that a large network is able to

control the dynamics of gene expression more precisely (Muller and Stelling 2009). Further work is required to distinguish between these possibilities.

While both ZCF15 and ZCF29 are required for full virulence and response to oxidative stress, Zcf29 more widely regulates expression, affecting over 20% of genes. In contrast, Zcf15 regulates only 2% of genes in the genome. It is possible that the recent duplication of Zcf15 partially explains the relatively smaller regulon of this transcription factor; ZCF15 has a closely related paralog, ZCF26, and is highly similar to additional ZCF genes (Figure 1). We hypothesize that the expansion of ZCF15 in C. albicans (with three paralogs) buffers the null mutant, such that a mild effect on virulence reflects deeper functionality. In the presence of oxidative stress, Zcf15 mobilizes the detoxification machinery, while Zcf29 downregulates energy expensive processes such as ribosome metabolism [e.g., S. cerevisiae regulates ribosome according to its estimate for the potential for growth. producing 40 ribosomes every second in exponential phase (Warner1999)]. Several profiling studies revealed a coordinated downregulation of genes involved in ribosome assembly upon adverse environmental conditions, including ROS (Moehle and Hinnebusch 1991; Li *et al.* 1999; Warner 1999; Gasch *et al.* 2000, 2001; Hughes *et al.* 2000). Genes in this pathway were upregulated in *zcf15/zcf15* null mutants (P = 0.1), suggesting that the mutant does not perceive any nutritional deficit. Thus, we hypothesize that the inability of the *zcf29/zcf29* mutant to downregulate an energy costly cellular process, such as ribosome biogenesis, compromises processes like DNA repair or cellular detoxification processes (Loar *et al.* 2004).

Three of the 37 Zcf15 DNA binding sites flank genes differentially expressed upon ZCF15 deletion in the absence of H₂O₂. Moreover, three of the 30 DNA binding sites that *ZCF15* recognizes in the presence of H_2O_2 are flanking genes that are H_2O_2 responsive and ZCF15 dependent. This result was not surprising, because (a) compared to Zcf29, the deletion of ZCF15 causes a significantly more modest transcriptional rewiring (168 differentially expressed genes for ZCF15 deletion vs. 1402 differentially expressed genes for ZCF29 (Figure 3A), and (b) it is not uncommon for TFs to bind regions that do not flank differentially expressed genes. For example, it is possible that many of the Zcf15-bound regions either regulate more distal transcripts, or that the binding is structural, i.e., maintains chromosome structure. The presence of distant-acting silencers or enhancers is also well documented in C. albicans (Tuch et al. 2010), and it is possible that some of the genes controlled by Zcf15 are located at genomic loci not flanking Zcf15-bound regions.

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