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 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 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 transcription factors.

INTRODUCTION

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 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 how cells function and respond to different environments, and approximately 4% of the *C. albicans* transcripts code for transcription factors (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) drug resistance (COWEN et al. 2002), as well as the transition from a commensal to a pathogenic lifestyle (LIU 2001). The zinc-finger 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 transcription factors represent a family of 82 Zn(II)$_2$Cys$_6$ 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 non-pathogenic 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 non-synonymous 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 transcription factors one at 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 non-pathogens (Figure 1A, 1B, 1C). ZCF15 is specifically expanded in pathogenic species, with 3 paralogs in C. albicans (Supplemental Figure 1) and in each of the pathogenic Candida, but absent in related non-pathogenic 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, C. lusitaniae) but is not present in phylogenetically related non-pathogenic species (S. cerevisiae, S. paradoxus or S. castelli) (Figure 1). Both ZCF15 and ZCF29 appear to be under strong purifying selection ($d_N/d_S$ 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 non-synonymous 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; JAIN et al. 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 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 supplementary tables. *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 **Supplemental Table 1**.

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 +/- ~650 bp was PCR amplified and co-transformed in *S. cerevisiae* BY4747 with BmgBI 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 *PmeI* 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 (Zinc Cluster Transcription Factor) designation. The Fungal Orthogroups tool within the *Candida* Genome Database was used to identify orthologs and paralogs of ZCFs (Figure 1B, 1C). 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 (dS>6 for both genes). Protein sequences were aligned using MUSCLE (EDGAR 2004) and converted to codon alignments with PAL2NAL (SUYAMA et al. 2006); dN/dS 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 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 *C. 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 manually scored 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, Inc.) 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 non-related 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°C in YPD, resuspended to OD = 1, serially diluted 1:5 in sterile water and spotted on agar plates containing SDS, Calcofluor White, NaCl, Sorbitol or paraquat at 0.04%, 20 µM, 0.5M, 1.5 M and 1mM respectively. For filamentation assays, strains were spotted on Spider media (1% nutrient broth, 1% D-mannitol, 2g K₂HPO₄) and incubated at 37°C for 7 days before being photographed. Ability to form biofilm was quantified using a previously described (REYNOLDS AND FINK 2001) protocol 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 2x10^6 cells were plated in 6-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 hours at 37°C, 5% CO₂ and then 1.3 x 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 (1 yeast cell per 15 RAW 264.7 cells). Plates were incubated at 37°C, 5% CO₂, overnight, scraped off each well and resuspended in 10 ml 0.02% Triton-X 100 (vol/vol) to osmotically lyse the macrophages. The solution was then spun down and the pellet resuspended in 1ml sterile water. From 1:10 serial dilutions, 100 μl of the 10^4 and 10^5 dilution were plated on YPD. After incubating the plates overnight at 30°C colonies were counted and percent killing determined by comparing cells counted in the plates with and without macrophages. Diphenyleneiodonium chloride (DPI) experiments were performed as described above but with the addition of 0.05 μM 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 OD₆₀₀=0.1 in YPD and allowed to grow for 4-6h to reach mid-exponential phase (OD₆₀₀=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 minutes and 15 minutes’ 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 H$_2$O$_2$ after 5 and 15 minutes as the transcriptional response of C. albicans to H$_2$O$_2$ has been shown to peak at 10-15 minutes post H$_2$O$_2$ 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 manufacturer’s instructions. Briefly, 5x10$^8$ total cells were re-suspended in 600 µl buffer RLT with β-mercaptoethanol (10 µl of β-mercaptoethanol for each 1 ml of RLT buffer) and transferred to 2ml bead beating tubes. 500 µl RNAse free Zirconia beads were added to the tubes and cells lysed by bead beating using a FastPrep instrument (MP Biomedicals). Tubes were centrifuged at 1000g for 5 minutes at 4 °C, the supernatant transferred to a gDNA eliminator column, and RNA isolated following the manufacturer 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 2µl of zinc fragmentation buffer (Life Technologies AM8740) to 18uL of mRNA and heating the samples at 70°C for 2 minutes. 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 rTdT 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 HIsq platform.

RNA-Seq reads were aligned to the C. albicans SC5314 reference genome (version A21-s02-m08-r01, downloaded from www.candidagenome.org) using TopHat (TRAPPELL 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 (Supplemental Figure 2 A, B and C, Supplemental Table 3). 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 ~74%, similar to previous studies (TUCH et al. 2010). For wild-type, zcf15/zcf15 and zcf29/zcf29 we obtained 49.3 million, 50.7 million 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 million, 66.6 million and 42.6 million for
samples extracted at $T_0$ (before the addition of $H_2O_2$), $T_5$ (5 minutes’ post addition of $H_2O_2$) and $T_{15}$ (15 minutes after the addition of $H_2O_2$) 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 (Supplemental Figure 2A). A small number of reads (~2-4%) aligned to intergenic region of the genome (possibly due to unannotated genes or sequencing error), and in a similar proportion found by others (DHAMGAYE *et al.* 2012). In addition, very few reads aligned to intronic regions (~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 (Supplemental Figure 2B) 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 x 27) correlation coefficients and summarized them as a heat map (Supplemental Figure 2C). 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 (Supplemental Figure 2C, box 1) and after the addition of H$_2$O$_2$ (Supplemental Figure 2C, box 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 (Supplemental Figure 3). 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 transcription factor (TF) was analyzed at 5 and 15 minutes after the exposure to 5mM H$_2$O$_2$ and compared to untreated controls. Upon H$_2$O$_2$ 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 *Candida 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 50x and 75x. Alignments were converted to
sorted BAM format using Samtools v0.1.9 (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 (npeak=300K). Peaks were next called on pooled replicates and pseudo-replicates 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 cross-correlation analysis of SPP to estimate the shift size parameter for each set of reads. Finally, we selected all peaks that were less than 300nt 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 log2 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 (~10% of the genome) ([Supplemental Table 2](#)) 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.](#)) ([PUKKILA-WORLEY et al. 2009](#)) also reviewed in ([ENGELMANN AND PUJOL 2010](#); [MARSH AND MAY 2012](#)). We identified 7 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* ([Supplemental Figure 4A](#)), 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* therefore validate our screening methods ([SCHALLER et al. 1999a](#); [SCHALLER et al. 1999b](#); [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 (Supplemental Figure 4B). 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 (Supplemental Figure 4B). 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 non-pathogenic fungi such as Saccharomyces cerevisiae (Figure 1A, Butler et al 2009). ZCF15 is specifically expanded in pathogenic species with 3 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 reactive oxygen species

To characterize the mechanism of the reduced virulence of zcf15/zcf15 and other Zcf family mutants, we phenotypically profiled the ten Zcf transcription factors that are conserved in pathogenic fungi but absent in non-pathogens. 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
ZCF29 is upregulated in the presence of H$_2$O$_2$ (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 Fungal Genetic Stock Center (Supplemental Table 1) 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 (Supplemental Figure 5) 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 ROS-deficient 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 wild-type when grown on E. 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.

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 (ARANA et al. 2007; LORENZ et al. 2004; WYSONG et al. 1998; 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 diphenyleneiodonium chloride (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 (H₂O₂). Transcriptional profiles obtained for each strain before and after the addition of H₂O₂ were dramatically different as suggested by their low degree of correlation (Supplemental Figure 2C, dashed blue rectangle). This indicates that H₂O₂ triggers a large transcriptional response within 5 minutes (T₅) that is even more dramatic after 15 minutes (T₁₅) as compared to control time point (T₀). Furthermore, biological replicate samples at T₅ were highly correlated with each other but poorly correlated with those at both T₀ and T₁₅ suggesting that the T₅ samples effectively captured an intermediate step in the H₂O₂ driven transcriptional rewiring obtained between T₀ and T₁₅.

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 hydrogen peroxide, 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 co-regulated between the zcf29/zcf29 and zcf15/zcf15 mutants have the same directionality of expression (Figure 3B). These 105 co-regulated genes include 50 upregulated and 55 downregulated genes (Figure 3B). Gene Set Enrichment Analysis (GSEA) revealed that genes required to maintain homeostasis of transition metals such as iron are enriched in this list of co-regulated 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$_2$O$_2$ to the a more toxic hydroxyl radical OH$^\cdot$ ROS (Winterbourn 1995). Within host niches, that 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-folds, HOM6 1.4-fold, MET2 1.8-fold and MET6 1.6-fold) while four of the five genes involved in threonine biosynthesis were significantly downregulated (HOM3 2.1-fold, HOM2-1.8 fold, HOM6 1.4-fold, 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 acid since all amino acid biosynthesis with the exception of arginine 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 8-fold and ARG5,6 6-fold). 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. 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 can’t 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 preferred source of energy for *C. albicans*, and 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 2 and 5-fold (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 $\text{CO}_2$ and $\text{H}_2\text{O}$ (Strijbis et al. 2008). *MIG1* is a transcription repressor that regulates carbon utilization (Zaragoza et al. 2000); the *S. cerevisiae* *MIG1* is a transcription factors 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 approximately 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 non-canonical 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 minutes, post exposure. We compared the transcriptional response triggered by sub-lethal concentration (5 mM) of H$_2$O$_2$. Hydrogen peroxide 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 (Supplemental Figure 6A) 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 is 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 non-canonical mechanisms.
To analyze the hydrogen peroxide responsive genes we clustered them into 4 groups based on their transcriptional responses (Supplemental Figure 6B and 6C) (ROBINSON et al. 2010). Groups 1 and 2 contain genes that are less (group 1) or more (group 2) downregulated by H$_2$O$_2$ 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$_2$O$_2$ treatment in the mutant compared to wild type.

To identify the biological functions enriched between wild type and zcf15/zcf15 upon H$_2$O$_2$ challenge we used GO term analysis for group 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 H$_2$O$_2$ may explain the hypersensitivity to ROS.

In the zcf29/zcf29 mutant, 110 genes were downregulated by H$_2$O$_2$ compared to wild type. These genes (group 1, Supplemental Figure 6C) 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 1.4x10^{-18}) suggesting that in the absence of ZCF29 many of these genes are misregulated, as they are not fully downregulated upon exposure to H$_2$O$_2$ (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, 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$_2$O$_2$ treatment SOD1, the superoxide dismutase that protects C. albicans from oxidative stress and is 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 functions results not only in its inability to grow on galactose as the sole carbon source but also to an increased H$_2$O$_2$
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 H₂O₂ 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 H₂O₂ 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 suggests 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 (Supplemental Figure 6). Chromatin immunoprecipitation coupled with DNA sequencing (ChIP-Seq) was used to identify the genomic sites bound by Zcf15 and Zcf29 (Nobile et al. 2009) (Methods) (Supplemental Figure 7, Supplemental Table 4). Samples from 5 and 15 minutes after the addition of 5mM H₂O₂ were compared to unexposed samples (T₀). In the absence of H₂O₂, we identified 151 and 141 regions bound by Zcf15 and Zcf29 respectively. In the presence of H₂O₂, we identified 93 and 210 regions bound by Zcf15 and Zcf29 respectively (IDR < 0.05). By focusing on regions that were ≤ 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, 6 of which were bound by both transcription factors (Figure 5A). The subset of these candidate regulated genes found in the presence or absence of H$_2$O$_2$ largely differed, as only 4 Zcf15 and 41 Zcf29 targets were detected in both conditions suggesting that Zcf15 and Zcf29 relocate to different genomic loci upon H$_2$O$_2$ exposure confirming our transcriptome analysis that showed regulations of different sets of genes in the presence or absence of H$_2$O$_2$. Orthology to Saccharomyces cerevisiae revealed three of these conserved targets as well as 9 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 hydrogen peroxide, 4 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 vesicle-associated 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 T$_0$ for Zcf15, and 2 distinct peaks upstream for Zcf29 - one that was present at T$_0$ and T$_5$, and the other that was present at all 3 time points (T$_0$, T$_5$ and T$_{15}$). 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$_2$O$_2$. Over half of the peaks for Zcf15 (18 of 32) and roughly a quarter of the peaks for
Zcf29 (19 of 84) were only found 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), 3 of which were identified at T₀ and T₅, and 1 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 hydrogen peroxide, 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 minutes of hydrogen peroxide exposure, Zcf29 binds specifically near 5 genes, of which 4 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 minutes of hydrogen peroxide exposure.

We performed de novo motif discovery and enrichment from the ChIP-Seq peaks, identifying distinct motifs for Zcf15 and Zcf29 bound sites (Supplemental Figure 8). 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 H₂O₂. 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 micro nutrient (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$_2$O$_2$ indicates that Zcf15 and Zcf29 relocate to different genomic targets, which is consistent with identification of multiple binding 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$_2$O$_2$.

**DISCUSSION**

Integrating metabolic inputs is crucial to *Candida 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; ENE et al. 2012b).

Here we describe a complex interconnected regulatory circuit driven by two fungal specific transcription factors that link the impact of metabolic and stress adaptations to virulence and immunogenicity. The Zinc Cluster Factor (ZCF) class of transcription factors is expanded within the lineage of pathogenic Candida (Figure 1A, 1B, 1C); however, their specific functional roles are largely not yet understood. In vitro phenotypic characterization revealed that ZCF15 and ZCF29 are required to respond to reactive oxygen species (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 the fungicidal effect on C. albicans. The zcf29/zcf29 mutant that 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 have 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 (Lorenz and Fink 2002); (Madhani and Fink 1998); (Brown et al. 2014). Together they regulate iron metabolism, another key micro-nutrient 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 micro-environment 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 commensal-pathogen, C. albicans co-evolves 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 (JAIN et al. 2013); (SCHUBERT et al. 2011); (MOGAVERO et al. 2011). 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$_2$O$_2$ exposure. Zcf29 plays a critical role in the H$_2$O$_2$ 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 controlled by Zcf15 and Zcf29, which play a critical role in resistance to reactive oxygen species 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* (SUEL *et al.* 2006); (DE HOON *et al.* 2010); (LOSICK AND STRAGIER 1992), 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 percent of genes. In contrast, Zcf15 regulates only two percent 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 3 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 (WARNER 1999)). Several profiling studies revealed a coordinated downregulation of genes involved in ribosome assembly upon adverse environmental conditions, including ROS (HUGHES et al. 2000); (GASCH et al. 2000; GASCH et al. 2001); (MOEHLE AND HINNEBUSCH 1991); (WARNER 1999); (LI et al. 1999). 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 down regulate 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, 3 of the 30 DNA binding sites that ZCF15 recognizes in the presence of H2O2 are flanking genes that are H2O2 responsive and ZCF15 dependent. This result wasn’t surprising because (a) compared to Zcf29, the deletion of ZCF15 causes a significantly more modest transcriptional rewiring (168 differentially expressed genes for ZCF15 deletion versus 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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Author contributions are as follows. R.P.R conceived and designed the general strategy of the genetic screen. WPI undergraduate student authors, B.L and K.P conducted the screen under the guidance of R.P.R. L.I, R.P.R and D.A.T designed subsequent experiments. L.I, R.A.F and T.D ran experiments and contributed to analysis under the guidance of R.P.R, C.A.C and D.A.T. G.W.B generated figures 1A and 3A. R.P.R, L.I, R.A.F, C.A.C and D.A.T drafted the manuscript.

FIGURE LEGENDS

Figure 1

Conservation of ZCF transcription factors and identification of two sensitive to oxidative stress. (A) The C. albicans genome encodes for approximately 240 Transcription factors (TFs). 82 listed in the figure belong to the special class of Zn(II)$_2$Cys$_6$ DNA binding proteins that are restricted to the fungal kingdom (not present in humans or plants) and expanded in pathogens. 35 of these are clustered (on the right) by phylogenetic
relatedness. These ZCFs (Zinc Cluster Transcription Factors) 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 3 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 1,000 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).

**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 μM 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 grey bars). Error bars indicate standard error. While macrophages treated with DPI, an agent that inhibits the production of ROS by inhibiting the activity of the NADPH oxidase (dark grey 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.

**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) 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 co-regulated genes. (C) The biosynthesis of the amino acid threonine (light blue), methionine (green), lysine, histidine, serine, leucine, tyrosine and phenylalanine (purple) are down regulated 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, 2 ubiquitin ligase genes, 6 genes involved in proteasome assembly, 6 genes directly involved in proteasome mediated degradation (amino and carboxyl peptidases), 1 gene involved in ubiquitin recycling (marked in brown). Differential gene expression was reported as log_{2}FC on the X-axis and statistical significance (–log10 (pvalue)) on the Y-axis. 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 it. 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 it.
Genes not statistically significantly differentially expressed are reported as black dots.

**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 x 10⁻⁴) and the downregulation of carbon metabolism (p-
value = 2.5 x 10⁻⁵). (B) ZCF29 is required for downregulation of ribosome biogenesis (p-
value 1.38 x 10⁻¹⁸) when exposed to H₂O₂.

**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
hydrogen peroxide. (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
canonical connections were inferred and an interconnected module identified that includes 3 of the
6 genes bound by both ZCFs.

**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.

**SUPPLEMENTAL FIGURES**

**Supplemental Figure 1**

The signature Zn(II)2Cys6 DNA binding motif (A) shown as a ribbon diagram. (B)
Syntenic analysis of ZCF15 locus in fungi. (C) Sequence alignment between the ZCF15
and its closest *C. albicans* paralogs that harbor the Zn(II)2Cys6 domain. Alignment using
*clustalW* indicates that paralogs ZCF26 shares 64.15% and ZCF25 shares 31.46%
identity with ZCF15 respectively. (D) The conservation histogram indicates that in
addition to the Zn(II)2Cys6 signature DNA binding motif (box1) that identifies these
transcription factors share homology that are both identifiable as domains (boxes 2-10)
or not.

**Supplemental Figure 2**

Quality control for RNA-Seq analysis (A) Total number of reads obtained were 154M, 2-8
M ready per sample. The large majority of the reads mapped to exonic region (~90-92%)
with only few reads mapping to intronic regions (3-4%) and intergenic regions (2-3%).
The low levels of intronic reads confirmed that our initial poly-A selection was effective in removing most of the genomic DNA. (B) The density of the reads observed in the ZCF15 (chromosome IV) and ZCF29 (chromosome VII) locus were visualized using the Integrative Genome Viewer (IGV). ZCF15 expression was not detected in any of the three zcf15/zcf15 biological replicates. ZCF29 expression was not detected in any of the three zcf29/zcf29- biological replicates. (C) Black boxes indicate a high degree of correlation between biological replicates. The blue dashed rectangle indicates a progressively decreasing degree of correlation between samples at T₀, T₅ and T₁₅, suggesting that the addition of H₂O₂ triggered a large transcriptional change across all strains. Yellow-dashed rectangle shows low degree of correlation between T₅ samples and both T₀ and T₁₅ samples. This evidence highlights that T₅ samples effectively captured an intermediate step in the H₂O₂ driven transcriptional change observed between T₀ and T₁₅.

Supplemental Figure 3

HA epitope tagging strategy for Zcf15 and Zcf29. HA-Arg4 insertion cassette is amplified from pFA- HA-Arg4 plasmid using primers containing ~70bp sequence homology for the region upstream and downstream of ZCF15 stop codon. Wildtype C. albicans is transformed with the HA-Arg4 insertion cassette that integrates by homologous recombination at the 3’ end of ZCF15 and consequently HA tagging Zcf15 at its C-terminus. Proper insertion of the HA-Arg4 insertion cassette was confirmed by diagnostic PCR targeting the new 5’ and 3’ junctions. Two independent transformants (T₁ and T₂) were confirmed. Proper expression of the Zcf15-HA tagged protein was confirmed by Western blot. Four independent transformants (T₁, T₂, T₃ and T₄) were tested and Cdc3-HA described here (Gerami-Nejad et al., 2009) was used as a positive control. (E→H)
Zcf29p was tagged using different primers but essentially the same logic described in A→D for Zcf15.

**Supplemental Figure 4**

An unbiased reverse genetic screen in live animals of 585 *C. albicans* mutants (88 mutants in protein kinases, 98 mutants in cell wall proteins, 162 mutants in transcription factors and 237 mutants in various other genes) was used to (A) identify 7 *C. albicans* mutants that showed a decrease in the deformed anal region (Dar) phenotype during *in vivo* infection of *C. elegans*. CMP1 encodes the catalytic subunit of calcineurin, and *C. albicans cmp1/cmp1* null mutants are unable to colonize the kidney in a mouse model of infection of disseminated candidiasis (*Bader et al. 2003*). *IFF11* is required for cell wall structure and null mutants show attenuated virulence in a mouse model of disseminated candidiasis (*Bates et al. 2007*). *Sap8* is a member of a large family of secreted aspartyl proteases that plays a crucial role in hydrolyzing host epithelial tissues, allowing *C. albicans* to penetrate and infect deeper tissues. *Sap8* in particular is upregulated an *in vitro* model of cutaneous candidiasis (*Naglik et al. 2003*). Identification of genes that regulate key processes like cell-wall architecture and host tissue invasion that have been shown to have a role in virulence (*Rubin-Bejerano et al. 2007; Wheeler et al. 2008; Liu and Filler 2011; Moyes et al. 2016) provide confidence in our screening methods (**Supplemental Figure 2A**). (B) Table summarizing the novel genes identified using a live animal screen for decreased virulence in *C. elegans*.

**Supplemental Figure 5**

Complementation strategies (A) *ZCF15* reintegrated in *zcf15/zcf15* knockout in the ectopic locus *LEU2*. The *ZCF15* open reading frame was amplified with primers containing 40 bp homologous sequences to *BmgBI* digested pSN105 (blue lines). *BmgBI*
digested pSN105 and ZCF15 were cotransformed into S. cerevisiae BY4741 to obtain plasmid pLI103. Plasmid pLI103 was digested with Pmel to liberate the ZCF15-ARG4 integrating cassette flanked by ~500 bp sequences homologous to the upstream and downstream region of LEU2 (green boxes). Digested pLI103 was transformed in C. albicans zcf15/zcf15 to introduce a wild-type copy of ZCF15 in LEU2 locus (chromosome VII). ZCF15 integration was confirmed by PCR checking the presence of the new 5’ and 3’ junctions at the LEU2 locus. (B) ZCF15 reintegrated in zcf15/zcf15 knockout at the endogenous locus. ZCF15 wild-type sequences (plus ~700 bp upstream of the gene) and ~700 bp sequence downstream of ZCF15 were cloned into pSN69 to obtain pLI102. This step was achieved using traditional cloning in E. coli and restriction enzymes BamHI, AflII, Xbal and XhoI. pLI102 was subsequently digested with BamHI and Xbal to liberate ZCF15-ARG4 which integrates at the ZCF15 endogenous locus on chromosome IV. Proper integration of ZCF15 was confirmed by PCR checking the presence of the new 5’ and 3’ junctions. (C) ZCF15 is required for wild-type resistance to reactive oxygen species generator paraquat. Wild-type, knockout or complemented strains were grown overnight, resuspended to OD = 1, serially diluted 1:5 and plated on either YPD or YPD + 1mM paraquat (D) C. elegans fights pathogens present in the intestinal lumen by producing ROS via bli-3 and contemporarily producing intracellular antioxidant via DAF-16 to protect its own tissues. (C) Kaplan-Meier survival curves showing that ZCF15 deletion reduces C. albicans’ ability to kill wild type worms. (E) Kaplan-Meier survival curve showing comparable killing kinetics between zcf15/zcf15, wild-type and complemented strain when ROS-deficient C. elegans bli-3(e767) were challenged.

Supplemental Figure 6
Differential expression of genes following hydrogen peroxide (H₂O₂) exposure. (A)
Regulation of key genes involved in global oxidative stress response – (I) the superoxide
detoxification pathway, *SOD1* and *SOD2* that convert superoxide ions to H₂O₂ which is subsequently converted into H₂O by *CAT1* (ii) the thioredoxins pathway, *TRR1*, *TRX1* and *TSA1* that acts as an electron donor to various peroxidases and other reeducates and helps in scavenging toxic ROS and (iii) the glutathione pathway functions as a cellular redox buffer scavenging toxic ROS through the action of *GLR1*, *orf19.86* and *orf19.4436* and (iv) *CAP1* are indistinguishable from wild type. The extent of upregulation is similar to previous reports (WANG et al. 2006), suggesting that the *zcf15/zcf15* and *zcf29/zcf29* mutants are not activating global oxidative stress response rather controlling the detoxifying processes via non-canonical novel pathways. (B) Group 1 represents genes downregulated by H₂O₂ in wild type but to a statistically significant lower degree in *zcf15/zcf15*. Group 2 represents genes downregulated in wild type but to a statistically significant higher degree in *zcf15/zcf15*. Using the same logic group 3 and 4 represents H₂O₂ induced genes but to a significant higher (group 3) or lower degree (group 4) in *zcf15/zcf15*. (C) Group 1 and 2 represent genes that are downregulated by H₂O₂ but to a statistically significant lower (group 1) or higher (group 2) degree in *zcf29/zcf29* compared to wild type. Group 3 and 4 represents genes that are upregulated by H₂O₂ but to a statistically significant higher (group 3) or lower (group 4) degree in *zcf29/zcf29*.

**Supplemental Figure 7**

Genomic location of Zcf15 and Zcf29 across hydrogen peroxide treatment across three time-points (T): T₀, T₅ and T₁₅. From the outside circles to the inner circles, we show (1) the length in megabases of the 8 nuclear chromosomes and mitochondrial (M) genome for *C. albicans*. (2-4) Normalized depth of coverage for time-points (T): T₀, T₅ and T₁₅, each of which is shown above boxes showing the locations of Zcf15 and Zcf29 (as peaks), and colored according to category as described in the central Venn diagram (red
= T₀, green=T₅, and blue=T₁₅). (5) Systematic names of genes with upstream peaks, highlighted in a color corresponding to the Venn diagram if different from T₀ (otherwise not highlighted). (6) Venn diagrams showing the number of peaks for each time-point, and their overlap.

**Supplemental Figure 8**

Motifs predicted from the Chip-Seq peaks for (A) ZCF15 and (B) ZCF29 respectively. (C) SFL1, a transcriptional repressor and activator from *S. cerevisiae*, shares similarity with ZCF29 motifs.

**SUPPLEMENTAL TABLES**

**Supplemental Table 1.**

Transposon insertion mutant library composition. A total of 585 mutants were screened: 88 mutants in protein kinases, 98 mutants in cell wall proteins, 162 mutants in transcription factors and 237 mutants in various other ORF.

**Supplemental Table 2.**

Strains obtained from Fungal Genetics Stock Center. * Two independent transformants tested.

**Supplemental Table 3.**

Summary of the RNA-seq data. Each sheet summarizes one RNA-seq comparison, as calculated by edgeR, including the following metrics: Gene/ORF symbol, log2 (fold
change), log2 (counts per million), likelihood ratio statistic, raw p-value, and False
Discovery Rate.

Supplemental Table 4.

Summary of the ChIP-seq data. The table lists the transcription factor that has been
immunoprecipitated (ZCF15, ZCF29 or both), the time point which a peak was identified
(t0, t5, t15), the genomic location of the peak (chromosome, start, stop), the upstream
gene, strand and the distance of the peak to that gene, the function of that gene, details
of any second upstream genes (if peak falls between 2 gene in opposite orientation),
and if significant log change in expression was identified from separate RNAseq
experiments.

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