A Novel Role for the Transcription Factor Cwt1p as a Negative Regulator of Nitrosative Stress in Candida albicans

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Abstract

The ability of Candida albicans to survive in the presence of nitrosative stress during the initial contact with the host immune system is crucial for its ability to colonize mammalian hosts. Thus, this fungus must activate robust mechanisms to neutralize and repair nitrosative-induced damage. Until now, very little was known regarding the regulatory circuits associated with reactive nitrogen species detoxification in fungi. To gain insight into the transcriptional regulatory networks controlling nitrosative stress response (NRS) in C. albicans a compilation of transcriptional regulator-defective mutants were screened. This led to the identification of Cwt1p as a negative regulator of NRS. By combining genome-wide location and expression analyses, we have characterized the Cwt1p regulon and demonstrated that Cwt1p is directly required for proper repression of the flavohemoglobin Yhb1p, a key NO-detoxification enzyme. Furthermore, Cwt1p operates both by activating and repressing genes of specific functions solicited upon NRS. Additionally, we used Gene Set Enrichment Analysis to reevaluate the C. albicans NRS-transcriptome and demonstrate a significant similarity with the transcriptional profiles of C. albicans interacting with phagocytic host-cells. In summary, we have characterized a novel negative regulator of NRS and bring new insights into the transcriptional regulatory network governing fungal NRS.

Introduction

Phagocytic cells such as macrophages and neutrophils form a part of the innate immune system and are considered as the first line of host defense against pathogens. These cells are able to ingest and subsequently kill infectious agents by producing a variety of toxic chemicals. The most important of these are antimicrobial peptides, nitric oxide (NO), the super oxide anion (O2−) and hydrogen peroxide (H2O2). In mammalian cells, NO is produced by a high-output form of nitric oxide synthase [1]. Upon phagocytosis, macrophages release reactive nitrogen species (RNS) and reactive nitrogen intermediates (RNI) into the phagolysosome to neutralize the engulfed pathogens. In addition to its direct antimicrobial role, NO reacts with the super oxide anion O2− to create the strong oxidant peroxynitrite (ONOO−), which has fungicidal activity [2].

Candida albicans is an opportunistic pathogen responsible for various non-life-threatening infections that can become very serious in immunocompromised patients. The ability of this commensal yeast to colonize numerous sites and organs requires that it can adapt rapidly to a variety of different environmental stresses such as reactive oxygen species (ROS) and RNS generated by phagocytic cells. The protective role of RNS against C. albicans was established in many infection models including murine oral candidiasis [3]. The ability of C. albicans to survive in the presence of nitrosative stress during the initial contact with the host immune systems is important for colonization. Thus, this fungus must activate robust mechanisms to detoxify RNS and repair NO-induced damage. Previous works had highlighted the role of the flavohemoglobin enzyme Yhb1p in NO detoxification through its action in converting the short NO radical to nitrate [4]. The C. albicans yhb1 homozygous mutant is hypersensitive to NO killing and exhibits a slightly reduced virulence in a tail vein model of disseminated candidiasis [5]. The transcription level of YHBI is highly induced by NO and its inducibility under nitrosative stress depends on the Zn(II)2-Cys6 transcription factor (TF) Cta4p [6,7]. A nitric oxide-responsive cis-regulatory element recognized by Cta4p in the promoter region of the flavohemoglobin YHBI gene has been identified.

Compared to mechanisms controlling the detoxification of ROS in fungi, very little is known regarding the transcriptional circuits and signaling pathways associated with RNS detoxification. To gain insight into these regulatory mechanisms in C. albicans we screened a compilation of mutants from various publicly available libraries [8–12], concentrating on mutants for genes encoding TFs
as well as components of chromatin remodeling and histone modification complexes. This comprehensive work led to the identification of a key TF that modulates the intensity of the nitrosative stress response (NSR) in *C. albicans*. Cwt1p was previously characterized as a regulator of cell wall damage and morphogenesis in *C. albicans* [13]. By combining genome-wide location and expression analyses, we have characterized the Cwt1p NSR-dependent regulon and demonstrate that this TF is required for proper repression of the flavohemoglobin Yhb1p which is a key NO-detoxification enzyme in *C. albicans* and other pathogenic fungi. This study also demonstrates that Cwt1p plays a role in both activating and repressing genes of specific functions solicited upon nitrosative stress response. Using Gene Set Enrichment Analysis (GSEA) we have revisited the role in both activating and repressing genes of specific functions solicited upon nitrosative stress response. Using Gene Set Enrichment Analysis (GSEA) we have revisited the role in both activating and repressing genes of specific functions solicited upon nitrosative stress response. Using Gene Set Enrichment Analysis (GSEA) we have revisited the role in both activating and repressing genes of specific functions solicited upon nitrosative stress response. Using Gene Set Enrichment Analysis (GSEA) we have revisited the role in both activating and repressing genes of specific functions solicited upon nitrosative stress response. Using Gene Set Enrichment Analysis (GSEA) we have revisited the enrichment analysis provided evidence for Cwt1p binding at 130 genes (Figure 1B) as expected, and this TF was also bound the promoter regions of the azole transporters Cdr1p and Mdr1p in addition to six genes (among fifteen) that were transcriptionally regulated in azole-resistant clinical strains overexpressing Cdr1p (orf19.7306, MDR1, IFD6, OIE2, orf19.7042 and orf19.7166) [16]. Cwt1p targets were notably enriched in other functional categories such as stress response, amino-acid metabolism, proteolysis, and the cell wall (Table S3). Cwt1p Binds the Promoter Region of the Nitric Oxide Dioxygenase YHB1 and Negatively Modulates its Transcription

Cwt1p was found to occupy the promoter of the flavohemoglobin/nitric oxide dioxygenase Yhb1p, a key enzyme of NO detoxification. Cwt1p was also detected at the YHB1 promoter using ChIP-qPCR, and the enrichment signal overlaps precisely with the carbon source response elements (CSREs) motif (AANYCCGA) recognized by the homologue of Cwt1p in *S. cerevisiae*, Rds2p [17,18] (Figure 4A). Cwt1p binding intensity at the YHB1 promoter was augmented significantly when Candida cells were challenged with DPTA NONOate compared to the control experiment suggesting a dynamic regulatory role of this TF during NSR. In order to assess the contribution of Cwt1p to the transcriptional regulation of this key NO-detoxifying enzyme, qPCR was used to evaluate the expression level of *YHB1* in wt and *cwt1* mutants under nitrosative stress condition at different time-points (5, 15, 60 and 180 min). As anticipated, the transcript levels of *YHB1* in wt cells were significantly induced when cells were challenged with the NO donor DPTA NONOate (Figure 4B). In the *cwt1* mutant, *YHB1* was induced similarly to the wt strain after exposure to DPTA NONOate for 5 min. However, the intensity of the NONOate transcriptional activation of *YHB1* was further enhanced in the *cwt1* mutant (Figure 4B) compared to the wt strain at 15 min, 60 min and 180 min. This demonstrates clearly that Cwt1p is a negative and direct transcriptional regulator of the NO-detoxifying enzyme *YHB1*. In the *cwt1* mutant, *YHB1* transcript level was the same as in wt after 5 min exposure to DPTA NONOate (Figure 4B). This suggests that Cwt1p-mediated repression occurs after the induction of *YHB1* and might happens to modulate *YHB1* inducibility by the TF Cta4p.

Gene Expression Analysis of Cwt1p Target Genes

As a preliminary step to assess the contribution of the TF Cwt1p on the NSR transcriptional program, NO-responsive genes were first identified by analyzing the transcriptional profile of wt *C. albicans* exposed to DPTA NONOate for 15 min (Table S3). In response to DPTA NONOate, the wt strain induced transcription of genes involved in iron transport/assimilation (*FET3*, *SIT*),
Figure 1. Growth inhibition assay by the nitric oxide donor DPTA NONOate. (A) Growth inhibition assay of selected mutant strains exposed to 0.3 and 1 mM DPTA NONOate during 3.5 hours. Growth inhibition rate was normalized relative to the 0 mM DPTA NONOate condition for each strain. Results are from three experiments. (B) Growth defect of cwt1 and cta4 strains was confirmed by an independent NO inhibition assay using different DPTA NONOate concentrations: 0, 0.1, 0.2, 0.3, 0.4, 0.5, and 1 mM. Growth of the wt (SN152) and cwt1/cwt1/CWT1 strains is also shown. Values are average of three replicates. Error bars are standard deviations of triplicates. The symbol (*) and (**) indicate a significant difference compared to the WT strain using t-test (P<0.0005 and P<0.0001, respectively).

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CTR2, FTR1, FRP6, FRP1, FTH1, HAP43, in addition to genes related to oxidative stress response (TTR1, ALO1, AHP1, HSP12). Transcript levels of genes involved in cell detoxification process such as the flavohemoglobin YHB1 and the glutaredoxin TTR1 were also induced (Table S5). Repressed transcripts were enriched in many functions, such as response to drug (PDR16, TPO3, FCR1,}

Figure 2. Confirmation of ChIP-chip by ChIP-Qpcr. In vivo occupancy of Cwt1p at ten different intergenic regions. Immunoprecipitated DNA was subjected to qPCR to validate Cwt1p binding at 100–200 bp regions surrounding the probes showing a significant binding ratio. SDs were based on data from two independent experiments. doi:10.1371/journal.pone.0043956.g002

Figure 3. Gene ontology analysis of Cwt1p-bound promoters. GO biological process, molecular function and cellular component annotations of Cwt1p bound promoters. P-values were calculated using hypergeometric distribution exploiting the Generic GO Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder). doi:10.1371/journal.pone.0043956.g003
Gene Set Enrichment Analysis (GSEA) was used to determine whether defined lists (or sets) of genes exhibit a statistically significant bias in their distribution within a ranked gene list [19]. Expectedly, as represented in Table 1 and Figure S1, genes upregulated and downregulated in response to DPTA NONOate exhibit a significant similarity with transcripts induced and repressed, respectively, during nitrosative stress in C. albicans.

Figure 4. Cwt1p bound the promoter region of the nitric oxide dioxygenase YHB1 and controls negatively its transcription. (A) Cwt1p is detected at the YHB1 promoter using tiled ChIP-qPCR, and the enrichment signal overlaps precisely its DNA-binding motif. Cwt1p occupancy was assessed in the presence and the absence of nitrosative stress. The nitric oxide-responsive element (NORE) recognized by the TF Cta4p is also shown. (B) Average expression of the nitric oxide dioxygenase YHB1 in response to 0.1 mM DPTA NONOate is shown in the wt and cwt1 strains in two independent biological replicates. Fold changes were estimated by using the coding sequence of the C. albicans ACT1 ORF as a reference. Fold enrichments of the tested coding sequences were estimated using the comparative ΔΔCt method.

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as reported previously by Hromatka et al. [7]. GSEA analysis of DPTA NONOate-induced genes further revealed a meaningful enrichment with transcripts upregulated in different in vivo infection models including a reconstituted human oral epithelium model [RHE] [14] and upon polymorphonuclear neutrophil-mediated phagocytosis [20] (Table 1 and S6).

Transcripts requiring Cwt1p for their proper regulation after exposure to DPTA NONOate were determined using a 1.5 fold cutoff and a statistical-significance analysis with an estimated false-discovery rate of 5% (Table S7). Results showed that Cwt1p is required for proper repression of 77 transcripts including genes involved in iron metabolism (PTIR, PAG10, HEM3 and RBT5), oxidative stress (GPX1, MDRI, SOD5 and DDR48) lipid catabolism (LIP5 and LIP3) and the flavohemoglobin YHBI (Table 2 and S7). Cwt1p was associated with the promoter of 14 genes among the 77 transcripts requiring this TF for their proper repression (Figure 5).

Cwt1p was also required for the accurate activation of transcripts related to hexose metabolism (GAL1, PDC11, SOU1, PGK1 and FBA1) and amino acid metabolism (MET15, SHM2, AAT1, MET14, GDH3, LAP3, GLT1 and GCV2). Using the same criteria as described above Cwt1p was found to bind 9 promoter regions among the 94 genes requiring Cwt1p for their proper activation (Figure 5, Table 2 and S7).

Discussion

Screening of Transcription Regulatory Mutant Libraries for Potential Modulators of Nitrosative Stress

Although the critical role of NSR in mediating the killing and inhibition of fungal pathogens such as C. albicans, A. fumigatus and C. neoformans [1] has been well established, little is known about the transcriptional regulators that control NO-detoxification process in fungi. In this work we have focused our investigation on the identification of transcriptional regulators controlling nitrosative stress in C. albicans using a comprehensive genome-wide screen of mutants for genes encoding TFs as well as components of chromatin remodeling and histone modification complexes. The compiled transcriptional regulatory mutant sets covered approximately 80% (170/213) of the TFs annotated in CGD and 40% (229/500) of proteins annotated to the GO term “regulation of gene expression”. This extensive investigation led to the identification of two transcriptional regulators, Cwt1p and Cta4p acting antagonistically in the control of nitrosative stress tolerance in C. albicans. Cta4p is a Zn(II)2-Cys6 TF that plays a dominant role in nitrosative stress resistance through a direct transcriptional activation the NO-detoxifying enzyme Yhb1p [6]. Cwt1p is also a member of zinc cluster TF family and is a homolog of S. cerevisiae Rds2p that plays a key role in regulating genes involved in gluconeogenesis, TCA and glyoxylate cycles [17]. CWT1 has been a subject of many functional characterizations in C. albicans, which showed that this TF is required for cell wall remodeling, morphogenesis and virulence [13,21]. Up to now, the contribution of Cwt1p, or its homologs Rds2p in the model organism S. cerevisiae, to the modulation of nitrosative stress has not been reported. Our work therefore uncovers a new regulator of fungal NSR.

Expanded Functional Role for the Cwt1p Transcription Factor in Fungi

Our genome-wide location profiling demonstrated that Cwt1p occupies the promoters of key gluconeogenesis specific genes, including PCK1 (Phosphoenolpyruvate carboxykinase) and PBP1 (Fructose-1,6-bisphosphatase), in addition to many genes implicated in the TCA cycle (KGD1, LSC2, FUM11 and HEM2) and gluconeogenesis/glycolysis common genes (PGK1, GPM2 and TPI1). Expression levels of some of those genes including the key gluconeogenic enzyme, Phosphoenolpyruvate carboxykinase PCK1 were also downregulated in the cwt1 strain suggesting that Cwt1p is directly mediating their transcriptional activation (Figure 6 and Table S7). The role of Cwt1p in gene expression regulation of

Table 1. Summary of Gene Set Enrichment Analysis of nitrosative stress response in C. albicans.

| Gene set | NES* |
|----------|------|
| Positive correlation with the profile | |
| Transcripts upregulated in response to ciclopirox [23] | 3.69 |
| Transcripts upregulated in Δsko1 [30] | 3.33 |
| Transcripts upregulated in response to nitric oxide [4] | 3.63 |
| Transcripts downregulated in Δdgg16 [31] | 3.74 |
| GO Process: Transition metal ion transport | 3.27 |
| Transcripts upregulated upon phagocytosis by polymorphonuclear neutrophils [32] | 3.21 |
| GO Process: iron transport | 2.49 |
| Transcripts downregulated in Δadc53 [33] | 3.93 |
| Tye7p-bound promoters [34] | 2.86 |
| Gal4p-bound promoters [34] | 3.23 |
| Transcripts upregulated in reconstituted human oral epithelium damaged by C. albicans [35] | 2.97 |
| Negative correlation with the profile | |
| Transcripts downregulated in response to nitric oxide | –3.46 |
| Transcripts differentially regulated during the S-G2 transition [33] | –3.03 |
| GO Process: mRNA transport | –2.56 |

*Normalized Enrichment Score (NES): a metric used to estimate statistical significance using an empirical phenotype-based permutation test procedure that preserves the complex correlation structure of the gene expression data [19]. NES adjust the Estimated Significance (ES) level to account for multiple hypothesis testing when an entire database of datasets is considered.

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Table 2. Gene expression analysis of Cwt1p target genes in response to nitrosative stress.

| Orf19 ID | Gene name | Description                                                                 | Expression ratio cwt1 vs wt | Cwt1p occupancy |
|----------|-----------|------------------------------------------------------------------------------|----------------------------|-----------------|
| **Upregulated transcripts** | | | | |
| **Iron metabolism** | | | | |
| orf19.7219 | FTR1 | High-affinity iron permease                                                  | 0.64                       | 1.21            |
| orf19.5674 | PGA10 | Plasma membrane protein involved in heme-iron utilization                    | 0.66                       | 1.12            |
| orf19.1742 | HEM3 | Hydroxymethylbilane synthase; catalyzes conversion of 4-porphobilinogen to 0.62 | 1.01                       |                 |
| orf19.4215 | FET34 | Protein similar to multicopper ferroxidase                                  | 0.66                       | 1.76            |
| orf19.5636 | RBT5 | GPI-anchored cell wall protein involved in hemoglobin utilization            | 0.48                       | 1.24            |
| **Oxidative stress** | | | | |
| orf19.87 | GPX1 | Putative thiol peroxidase                                                   | 0.66                       | 1.55            |
| orf19.7111.1 | SOD3 | Cytosolic efflux pump of plasma membrane; member of the MDR family           | 0.08                       | 1.00            |
| orf19.3461 | – | Predicted manganese-containing superoxide dismutase, involved in protection against oxidative stress | 0.61                       | 1.40            |
| orf19.4082 | DDR48 | Immunogenic stress-associated protein                                       | 0.44                       | 1.03            |
| **Lipid metabolism** | | | | |
| orf19.5179 | LIP5 | Secreted lipase                                                          | 0.12                       | 1.23            |
| orf19.4856 | LIP3 | Secreted lipase                                                          | 0.61                       | 1.11            |
| orf19.137 | CST26 | Protein required for incorporation of stearic acid into phosphatidylinositol | 0.09                       | 1.57            |
| orf19.3203 | YHA1 | Nitric oxide dioxygenase, acts in nitric oxide scavenging/detoxification    | 4.73                       | 11.16           |
| **Downregulated transcripts** | | | | |
| **Hexose metabolism** | | | | |
| orf19.3672 | GAL10 | UDP-glucose 4-epimerase, required for galactose utilization                  | 1.78                       | 1.02            |
| orf19.3681 | PGK1 | Phosphoglycerate kinase; enzyme of glycolysis                               | 1.55                       | 2.12            |
| orf19.4217 | FBA1 | Putative fructose-bisphosphate aldolase; enzyme of glycolysis               | 1.58                       | 1.55            |
| orf19.2877 | PDC11 | Protein similar to pyruvate decarboxylase                                   | 1.69                       | 1.54            |
| orf19.2896 | SOU1 | Enzyme involved in utilization of L-sorbose                                 | 1.85                       | 1.07            |
| **Amino acid metabolism** | | | | |
| orf19.5645 | MET15 | O-acetylhomoserine O-acetyls erine sulfhydrlyase; involved in sulfur amino acid biosynthesis | 1.94                       | 1.20            |
| orf19.5750 | SHM2 | Cyttoplasmic serine hydroxymethyltransferase                               | 1.55                       | 1.21            |
| orf19.3554 | AAT1 | Protein described as aspartate aminotransferase                            | 1.62                       | 3.56            |
| orf19.9467 | MET14 | Putative adenylylsulfate kinase; predicted role in sulfur metabolism        | 1.59                       | 1.00            |
| orf19.4716 | GDH3 | Protein described as similar to NADP-glutamate dehydrogenase                | 4.10                       | 1.00            |
| orf19.539 | LAP3 | Protein described as an aminopeptidase                                      | 1.53                       | 1.14            |
| orf19.6257 | GLT1 | Putative glutamate synthase                                                 | 1.84                       | 1.00            |
| orf19.385 | GCY2 | Putative protein of glycine catabolism                                     | 1.63                       | 1.01            |
| orf19.1063 | FMP64 | Putative protein similar to high-affinity phosphate transporters            | 1.87                       | 1.07            |
| orf19.2454 | PHO87 | Protein similar to phosphate permeases                                     | 1.52                       | 1.00            |
| **Phosphate transport** | | | | |
| orf19.1274 | FMP44 | Putative protein similar to high-affinity phosphate transporters            | 1.87                       | 1.07            |
| orf19.3656 | NTE1 | Putative patatin-like phospholipase                                        | 1.59                       | 1.00            |
| orf19.6257 | PLB1 | Phospholipase B                                                          | 1.52                       | 1.04            |
| orf19.7585 | INO1 | Inositol-1-phosphate synthase; enzyme of inositol biosynthesis             | 1.55                       | 1.49            |
| orf19.6459 | DPP3 | Protein similar to S. cerevisiae pyrophosphate phosphatase Dpp1p; required 1.67 | 1.10                       |                 |

Selected GO categories are shown for both down- and upregulated transcripts. Cwt1p binding ratio is also indicated.
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carbohydrate metabolism thus appears to be conserved between C. albicans and S. cerevisiae [17]. However, Cwt1p was found to be associated with the promoters of genes related to pyruvate metabolism (PDB1, LAT1, MAEI and PDX1) specifically in C. albicans. The list of Cwt1p promoter targets was also enriched for genes from a variety of GO categories that has not previously been related to Rds2p, including translation, proteolysis, vesicle-mediated transport and amino acid metabolism (Table S3). Another target of Cwt1p in C. albicans includes YHB1, encoding the key NO-detoxifying enzyme flavohemoglobin/nitric oxide dioxygenase. Our investigations demonstrated that Cwt1p is a negative modulator of nitrosative stress resistance through a direct transcriptional control of YHB1. The role of Cwt1p in mediating NSR is thus novel and unique to C. albicans since S. cerevisiae rds2 mutant didn’t show any growth alteration in the presence of different DPTA NONOate concentrations (data not shown).

Transcriptional Regulatory Network Mediating Nitrosative Stress Response in C. albicans

The transcriptional response to nitrosative stress has been well elucidated in several fungi including C. albicans [7]. Generally, in response to DPTA NONOate Candida cells upregulates genes involved in the oxidative stress response, iron uptake and sulfur assimilation. In our investigation we reexamined the C. albicans nitrosative stress transcriptome using GSEA. This method ranks genes according to their changes in transcript abundance and compares them to a large collection (5689 gene sets) of expression profiling, ChIP-chip, and GO categories data in addition to different S. cerevisiae synthetic genetic interactions and protein-protein interaction datasets. This analysis revealed an interesting and unexpected correlation with the cichlopox olamine expression profiling data (Table 1 and Figure S1). Cyclopox olamine is an anti-infective agent that seems to act as an iron chelator [22]. Similarly, as reported in this work for the DPTA NONOate, cyclopox olamine induces the transcription of many genes involved in iron uptake and oxidative stress [23] suggesting that this compound might cause nitrosative stress. This hypothesis is buttressed by the fact that, like the DPTA NONOate, cyclopox olamine has an ethanolamine chemical group that is a potential nitric oxide donor. Additional support of this assumption came from recent work undertaken in rats which demonstrated that perfusion with cyclopox significantly increased the endogenous level of NO [24].

Intriguingly, nitrosative stress responsive genes exhibit a convincing correlation with the transcriptional programs of C. albicans interacting with host-cells, such as polymorphonuclear neutrophils [14]. This resemblance strongly supports the hypothesis that immune cells, such as neutrophils, inhibit C. albicans growth through the release of nitric oxide radicals. Taking into consideration the resistance of cwt1 to NO radicals, this mutant should have a beneficial capacity to circumvent the immune system defense and an in vivo fitness gain. However, previous works undertaken in mouse model have shown that the virulence of cwt1 mutant was similar to its parental background strain [13], suggesting a minor contribution of NO-resistance to Candida pathogenicity in this systemic infection model.

In conclusion, we have characterized a new transcriptional regulator of NSR, which act as a cis-repressor of the key NO-detoxifying enzyme Yhb1p as well as other NO-responsive transcripts. Cwt1p is also required a proper activation of many NO-responsive genes which are its direct targets implying that this TF, as reported for its homolog Rds2p in S. cerevisiae [17], behaves both as a repressor and activator. Cwt1p was also associated with the promoter of transcriptional regulators including different TFs (Ze1lp, Sut2p, Cas1p, Lys14p) related to different biological functions and two transcriptional repressors Mig1p and Nrg1p controlling genes involved in carbohydrate metabolism. This suggests that Cwt1p is a high-level hierarchical regulator controlling different biological processes in C. albicans.

Materials and Methods

C. albicans Strain Construction, Growth Media and Conditions

For general propagation and maintenance conditions, the strains were cultured at 30°C in yeast-peptone-dextrose (YPD) medium supplemented with uridine (2% Bacto peptone, 1% yeast extract, 2% dextrose, and 50 µg/ml uridine). For gene expression profiling of cells treated with Dipropylethramine NONOate (DPTA NONOate; Cayman chemical), saturated overnight cultures of the wild type SN152 (arg4A/arg4A, leu2A/LEU2, his1A/HIS1, URA3/ura3, IRO1/iro1D) and cwt1 mutant (Homann TF collection [8]) were diluted to a starting optical density at 600 nm (OD600) of 0.1 in 100 ml fresh YPD buffered with 50 mM sodium phosphate pH 7.3, grown at 30°C to an OD600 of 0.8 and then split into two 30 ml cultures. DPTA NONOate was added to the experimental culture to a final

Figure 5. Cwt1p is a direct regulator of nitrosative stress responsive-genes. Relationship between Cwt1p-bound genes and genes showing altered expression in the cwt1 mutant challenged by 0.1 mM DPTA NONOate during 15 min. The symbol (*) and (**) indicate a significant difference compared to the WT strain using t-test (P<0.0005 and P<0.0001, respectively). doi:10.1371/journal.pone.0043956.g005
concentration of 0.1 mM. An equal volume of YPD buffered with 50 mM sodium phosphate pH 7.3 was added to the control culture. For the profiling experiment using microarray, the cultures were incubated for 15 min. Different time-points (5, 15, 60 and 180 min) were considered for the qPCR experiment assessing the transcript level of YHB1. Cells were harvested by centrifugation and stored at $-80^\circ\text{C}$.

For reintegration experiments, the CWT1 gene was reintegrated into the null mutant cwt1 strain from the Homann TF deletion library [8]. The wild-type CWT1 gene was amplified from genomic DNA using oligonucleotides Ctw1RevF1and Ctw1RevR1 (Table S8) and Expand high fidelity polymerase (Roche). The PCR fragment was digested with restriction enzymes SalI and MluI and cloned in the same sites of the CIp30 vector [25]. The plasmid was sequenced to confirm the integrity of the CWT1 gene. Plasmid CIp30-CWT1 was digested with the StuI restriction enzyme and used to transform the cwt1 mutant strain. The arginine-positive colonies were analyzed by PCR and the obtained wild-type fragment confirmed the reintegration of the CWT1 gene.

Growth Inhibition Assays

NO inhibition assay was performed as described by [6]. Library screening was performed at two DPTA NONOate concentrations: 0.3 mM and 1 mM. Growth inhibition rate was normalized relative to the 0 mM DPTA NONOate condition and was compared to their respective parental strain (SN152 for Homann's deletion library [8], BWP17 for Sanglard's insertion library, DAY185 for Mitchell's insertion library [12] and SN250 for Noble's deletion library [9]). The growth defect of the cwt1 and cta4 strains was confirmed by an independent NO inhibition assay using different DPTA NONOate concentrations: 0, 0.1, 0.2, 0.3, 0.4, 0.5, and 1 mM.

Quantitative Real Time PCR

Cells for gene expression analysis by qPCR were prepared as described for the microarray experiment except that cultures were incubated with DPTA NONOate at different time-points (5, 15, 60 and 180 min). cDNA was synthesized from 2 µg of total RNA using the reverse-transcription system (50 mM Tris-HCl, 75 mM KCl, 5 mM DTT, 3 mM MgCl2, 400 nM oligo(dT)15, 20 ng random octamers, 0.5 mM dNTPs, 200 units Superscript III reverse transcriptase; Invitrogen). The mixture was incubated for 30 min at 50°C. cDNAs were then treated with 2U of RNase H (Promega) for 20 min at 37°C followed by heat inactivation of the enzyme at 80°C for 10 min. Aliquots were used for qPCR, which was performed using the Mx3000P QPCR System (Agilent) with the QuantiTect SYBR Green PCR master mix (Qiagen). Cycling was 10 min at 95°C followed by 40 cycles (95°C, 10 s; 58°C, 15 s; 72°C, 15 s). Samples were done in triplicate and means were used for calculations. Fold changes were estimated by using the coding sequence of the C. albicans ACT1

Figure 6. Cwt1p occupancy of gluconeogenesis, pyruvate metabolic and TCA cycle genes. Cwt1p binding ratio and transcription level of genes are shown in blue and green, respectively. Genes bound and requiring Cwt1p for their activation were highlighted in red. doi:10.1371/journal.pone.0043956.g006
ORF as a reference. Fold enrichments of the tested coding sequences were estimated using the comparative ΔΔCt method [26]. Primer sequences used for this analysis are summarized in Table S8.

ChIP-ChIP and ChIP-qPCR

CWT1 was TAP-tagged in vivo with a TAP-URA3 PCR product in BWP17 strain as described by [27]. Transformants were selected on YPD-ura plates and correct integration of the TAP-tag was checked by PCR and sequencing. CWT1-TAP expressed in BWP17 strain was fully functional based on complementation of the nitrosative stress sensitivity phenotype. Deleting the non-tagged allele in the CWT1-TAP strain revealed that this strain has a comparable sensitivity to the single knockout strain and even to the parental strain. ChIP-chip was performed as previously described [27,28] with few modifications. Briefly, cells were grown to an optical density at 600 nm of 1.8 in 45 ml of YPD. Tiling arrays were co-hybridized with tagged immunoprecipitated (Cy3-labeled) and mock immunoprecipitated (untagged BWP17 strain; Cy3-labeled) DNA samples. Binding locations were determined in duplicate ChIP-chip experiments using microarray containing 5449 70-mer oligonucleotides representative of all intergenic regions. Data handling and analysis were carried out using Genespring v.7.3 (Agilent Technologies, Palo Alto, CA). Statistical analysis using the Welch t test with a false-discovery rate of 5% and 1.6 fold enrichment cutoff was used. For qPCR validations, in vivo TAP-tagged Cwt1p was ChIPed as described for chip-chip. PCR reactions were performed exactly as for gene expression analysis. Enrichments of tested promoter sequences were estimated by using the coding sequence of the C. albicans ACT1 ORF as a reference. Oligonucleotide sequences are given in Table S8.

ChIP-qPCR was performed using 1 ng of TAP-ChIPed DNA or total genomic DNA extracted from the whole cell extract (WCE). Cycling was for 15 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s. All samples were tested in duplicates and means were used for further calculations.

RNA Preparation

Total RNA was extracted as described in [29] with few modifications. Briefly, samples stored at −80 °C were placed on ice and RNeasy buffer RLT was added to pellets at a ratio of 1:1 [vol/vol] buffer/pellet. The suspended pellet was placed back on ice and divided into 1 ml aliquots in 2 ml screw cap microcentrifuge tubes containing 0.6 ml of 3 mm diameter acid-washed glass beads. Samples were homogenized 5 times, 1 min each in a FastPrep®-24 bead beater for 60 s at 6 m/s. After the homogenization the Qiagen RNeasy protocol was followed as recommended. Total RNA samples were eluted in RNase free H2O. RNA quality and integrity were assessed using an Agilent 2100 bioanalyzer.

Microarray Hybridization and Processing

cDNA labeling and microarray production were performed as described by Nantel et al. [30]. Briefly, 14 μg of total RNA was reverse transcribed using 9 ng of oligo(dT)21 in the presence of Cy3 or Cy5-dCTP (Invitrogen) and 400 U of Superscript III reverse transcriptase (Invitrogen) in a 40 μl reaction. After cDNA synthesis, template RNA was degraded by adding 2.5 units RNase H (Promega) and 1 μg RNase A (Pharmacia) followed by incubation for 15 min at 37 °C. The labeled cDNAs were purified with QIAquick PCR Purification Kit (Qiagen). DNA microarrays were processed and analyzed as above for ChIP-chip experiment. NO-responsive transcripts and genes requiring Cwt1p for their proper regulation were determined using the Welch t-test with a false-discovery rate of 5% and 1.5 fold enrichment cutoff.

Gene Set Enrichment Analysis (GSEA)

Gene Set Enrichment Analysis (GSEA), was used to determine whether defined lists (or sets) of genes exhibit a statistically significant bias in their distribution within a ranked gene list (see http://www.broadinstitute.org/gsea/for details [19]). This required initially the construction of an extensive gene set and annotation database using publicly available data from CGD, SGD and BioGRID, together with transcription factor binding data from all currently published ChIP-chip experiments, our own TF motif database, lists of modulated genes from both transcriptional profiling experiments, and genetic-association data obtained from SGA screens measuring cell growth.

MEME Analysis

For de novo identification of the consensus-binding site, the sequences were analyzed with the Multiple EM for Motif Elucidation (MEME) program (http://meme.sdsu.edu/meme/cgi-bin/meme.cgi). Sequences of 300 bp upstream the ATG of 17 Cwt1p targets (using a 3 fold binding enrichment cutoff) were extracted and used for min-motif detection. The motifs were allowed to have any sequence and length (between 6 and 20 bp) and could be present anywhere in the sequence.

Supporting Information

Figure S1

(TIF)
Table S1

Results of the primary screen of C. albicans mutant collections using 0.3 mM and 1 mM of DPTA NONOate.
(XLS)
Table S2

ChIP-chip datasets showing DNA loci occupied by Cwt1p.
(XLSX)
Table S3

Gene Ontology analysis of Cwt1p-targets.
(XLSX)
Table S4

The five most significant motifs in the core promoter sequences from −300 bp to 0 bp relative to the ATG codon, of 17 Cwt1p-bound promoter regions (top ranked Cwt1p targets) as identified by the MEME algorithm.
(DOCX)
Table S5

NO-responsive genes identified by analyzing the transcriptional profile of wt C. albicans exposed to DPTA NONOate for 15 min.
(XLSX)
Table S6

GSEA analysis of DPTA NONOate-differentially expressed in the wt strain.
(XLSX)
Table S7

Genes differentially expressed in cwt1 mutant after treatment with 0.1 mM DPTA NONOate during 15 min.
(XLS)
Table S8

List of primers used in this study.
(DOCX)

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References

1. Brown AJ, Haynes K, Quinn J (2009) Nitrosative and oxidative stress responses in fungal pathogenicity. Curr Opin Microbiol 12: 384–391.

2. Nathan C, Shoham MU (2000) Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci U S A 97: 8414–8416.

3. Elahi S, Pang G, Ashman RB, Clancy R (2001) Nitric oxide-enhanced resistance to oral candidiasis. Immunology 104: 447–454.

4. Gardner PR, Gardner AM, Martin LA, Salzman AL (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. Proc Natl Acad Sci U S A 95: 10378–10383.

5. Ullmann BD, Myers H, Chiranand W, Lazzell AL, Zhao Q, et al. (2004) A phenotypic transcription factor mediates induction of nitrosative stress response in Candida albicans. Eukaryot Cell 3: 713–723.

6. Chiranand W, McLeod I, Zhou H, Lynn JJ, Vega LA, et al. (2008) CTA4 transcription factor mediates induction of nitrosative stress response in Candida albicans. Eukaryot Cell 7: 260–278.

7. Hromatka BS, Noble SM, Johnson AD (2005) Transcriptional response of Candida albicans to nitric oxide and the role of the YHB1 gene in nitrosative stress and virulence. Mol Biol Cell 16: 4814–4826.

8. Homann OR, Dea J, Noble SM, Johnson AD (2009) A phenotypic profile of the Candida albicans regulatory network. PLoS Genet 5: e1000785.

9. Noble SM, French S, Kohn LA, Chen V, Johnson AD (2010) Systematic screening of a Candida albicans homozygous deletion library decouples morphogenic switching and pathogenicity. Nat Genet 42: 590–598.

10. Epp E, Vanier G, Harscud B, Lee AY, Jansen G, et al. (2010) Reverse genetics in Candida albicans predicts ARF cycling is essential for drug resistance and virulence. PLoS Pathog 6: e1000739.

11. Bruno VM, Kalachikov S, Subaran R, Noble CJ, Kyrtatas G, et al. (2006) Control of the C. albicans cell wall damage response by transcriptional regulator Ca5. PLoS Pathog 2: e21.

12. Noble CJ, Mitchell AP (2005) Regulation of cell-surface genes and biofilm formation by the C. albicans transcription factor Bcr1p. Curr Biol 15: 1150–1155.

13. Moreno I, Martinez-Esparza M, Laforet LC, Sentandreu R, Ernst JF, et al. (2010) Dosage-dependent roles of the CWT1 transcription factor for cell wall architecture, morphogenesis, drug sensitivity and virulence in Candida albicans. Yeast 27: 77–87.

14. Fradin C, De Groot P, MacCallum D, Schaller M, Klis F, et al. (2005) Granulocytes govern the transcriptional response, morphology and proliferation of Candida albicans in human blood. Mol Microbiol 56: 397–415.

15. Bailey TL, Elkan C (1994) Fitting a mixture model by expectation maximization. Curr Opin Struct Biol 4: 754–760.

16. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, et al. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102: 15545–15550.

17. Spiering MJ, Moran GP, Chauvel M, MacCallum DM, Higgins J, et al. (2010) Comparative transcript profiling of Candida albicans and Candida dubliniensis identifies SFL2, a C. albicans gene required for virulence in a reconstituted epithelial infection model. Eukaryot Cell 9: 251–263.

18. Moreno I, Pedreno Y, Maicas S, Sentandreu R, Herrero E, et al. (2003) Characterization of a Candida albicans gene encoding a putative transcription factor required for cell wall integrity. FEMS Microbiol Lett 226: 159–167.

19. Abrams BR, Hanel H, Hoehler T (1991) Ciclopirox olamine: a hydroxypyridone antifungal agent. Clin Dermatol 9: 471–477.

20. Lee RE, Liu TT, Barker KS, Lee RE, Rogers PD (2005) Genome-wide expression profiling of the response to ciclopirox olamine in Candida albicans. J Antimicrob Chemother 55: 655–662.

21. Fang ML, Li M, Lahiri S (2007) Increased endogenous nitric oxide release by iron chelation and purinergic activation in the rat carotid body. Open Biochem J 1: 1–6.

22. Dennison PM, Ramsdale M, Manson CL, Brown AJ (2005) Gene disruption in Candida albicans using a synthetic, codon-optimized Cre-loxP system. Fungal Genet Biol 42: 737–748.

23. Guilleminette T, Sellam A, Simonneau P (2004) Analysis of a nonribosomal peptide synthetase gene from Alternaria brassicae and flanking genomic sequences. Cur Genet 45: 214–224.

24. Lavoie H, Sellam A, Askew C, Nantel A, Whiteway M (2006) A toolbox for epitope-tagging and genome-wide location analysis in Candida albicans. BMC Genomics 9: 578.

25. Sellam A, Tebbbi F, Nantel A (2009) Role of Ndt80p in sterol metabolism regulation and asexual resistance in Candida albicans. Eukaryot Cell 8: 1174–1183.

26. Nantel A, Askew C, Epp E, Lavoie H, Whiteway M, et al. (2009) Genome-wide mapping of the coactivator Ada2p yields insight into the functional roles of SAGA/ADA complex in Candida albicans. Mol Biol Cell 20: 2389–2400.

27. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, et al. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102: 15545–15550.

28. Cote P, Hogues H, Whiteway M (2006) Microarrays for Studying Gene expression profiling of the response to ciclopirox olamine in Candida albicans. Eukaryot Cell 4: 890–899.

29. Barwell KJ, Boysen JH, Xu W, Mitchell AP (2005) Relationship of DFG16 to cell wall integrity in Candida albicans. Eukaryot Cell 4: 713–723.

30. Navarathna DH, Amarnath S, Akis MSL, Nayak D, Roberts DD (2012) Ciclopirox olamine: a hydroxypyridone antifungal agent. Clin Dermatol 9: 471–477.

31. Abrams BR, Hanel H, Hoehler T (1991) Ciclopirox olamine: a hydroxypyridone antifungal agent. Clin Dermatol 9: 471–477.

32. Lee RE, Liu TT, Barker KS, Lee RE, Rogers PD (2005) Genome-wide expression profiling of the response to ciclopirox olamine in Candida albicans. J Antimicrob Chemother 55: 655–662.

33. Baechevis C, Nantel A, Whiteway M (2006) Microarrays for Studying Gene expression profiling of the response to ciclopirox olamine in Candida albicans. J Antimicrob Chemother 55: 655–662.

34. Bachewich C, Nantel A, Whiteway M (2005) Cell cycle arrest during S or M phase generates pulsed growth via distinct signals in Candida albicans. Mol Microbiol 57: 942–959.

35. Barwell KJ, Boysen JH, Xu W, Mitchell AP (2005) Relationship of DFG16 to the RIM101p pH response pathway in Saccharomyces cerevisiae and Candida albicans. Eukaryot Cell 4: 890–899.

Author Contributions

Conceived and designed the experiments: AS FT. Analyzed the data: AS FT MW AN. Wrote the paper: AS FT.