Bioinformatic approach to the model of nitrosative stress response in *Candida glabrata*.

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Abstract— *Candida glabrata* is an opportunistic pathogen that produce systemic infection in immune-compromized patients. This yeast can internalize into the macrophage by means of phagocytosis, once this yeast is engulfed it changes the internal pH from acid to alkaline, to evade the lytic activity of phagosome, and overcomes other stresses such as oxidative and nitrosative stress. Linde et al. (2015) performed differentially expressed gene analysis under alkaline stress and nitrosative stress caused by thio-nitrosoglutathione (GSNO) in *C. glabrata*. They found that transcriptional profile of this pathogen yeast was very different comparing it to *Saccharomyces cerevisiae* and *Candida albicans* transcriptional profile observed under nitrosative stress. It is noteworthy that ribosome biogenesis genes were up-regulated under nitrosative stress in *C. glabrata*, inasmuch as it is expected that under a stress condition, these genes should be repressed. Based on gene set enrichment analysis we found that *C. glabrata* seems not to be under nitrosative stress except for the over-expression of the *YHB1* gene, a flavohemoglobin that protects this yeast from the damage caused by nitric oxide. We found, as it is expected, DNA motifs similar to PAC and RRPE elements enriched in pormoter sequences of up-regulated genes under nitrosative stress and we also found a motif very similar to MluI DNA motif recognized by Mbp1, a transcription factor involved in up-regulating genes of DNA synthesis and cell cycle progression. Accordingly, we found many genes with at least one MluI DNA motif on its promoter to be significantly expressed and the enriched GO terms are related to DNA synthesis and cell cycle progression. With these results we propose an hypothesis that protein and DNA synthesis is activated by the presence of GSNO or the nitrates produced by the flavohemoglobin Yhb1, and that the expression of this protein is enough to overcome the nitrosative stress caused by GSNO in *C. glabrata*. Regarding alkaline stress response in *C. glabrata*, it is similar to the alkaline stress response observed in *S. cerevisiae*. Transcriptional profile suggests that the pathogen yeast is under a nutritional stress condition and iron homeostasis unbalance.

Index Terms— Alkaline stress, *Candida glabrata*, Mbp1 DNA motif, Nitrosative stress, Pathogenic yeasts.

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1 Introduction

The yeast *Candida glabrata* is an opportunistic pathogen of humans, and has developed different strategies to evade the host's immune system. One of these strategies involves the internalization of the pathogen by macrophage phagocytosis. Once it is inside, this yeast modulates the internal pH of the phagosome turning it from acidic to alkaline, evading the lytic activity and phagosome maturation [1], [2]. Additionally, it is capable to withstanding other stresses like the oxidative, carbon-source depletion [3], [4] and nitrosative stress [5]. It can also survive, grow and proliferate inside the host cell by the support of autophagy [6], [7]. In spite of all this knowledge, little is known about how *C. glabrata* responds to the nitrosative stress it faces when interacts with its host provoked by nitric oxide reactive nitrogen species donated by the compound S-nitroso gluthatione [5].

The closely related model yeast *Saccharomyces cerevisiae* has been widely used to study the response to different stresses like, oxidative, heat shock, membrane perturbation, metal-ions homeostasis changes, carbon-source depletion and nitrosative stress. When *Saccharomyces cerevisiae* faces a wide variety of stresses, a common response is triggered, it is the negative regulation of genes involved in ribosome biogenesis (RiBi) and protein synthesis [8], [9], [10]. The functional role of transcriptional factors Dot6 and Tod6 in negatively-regulating Ribi genes is extensively documented in *S. cerevisiae*. These factors are activated in response to several stresses [11], [12]. Promoter regions of Ribi genes are enriched in PAC DNA motif (CGATGAG) which is recognized by the repressors Dot6, Tod6 and Stb3, and the ribosomal RNA processing element (RRPE) (GAAAATTT), which is recognized by the transcriptional activator Sfp1, although Sfp1 binding to RRPE DNA motif is not direct [12], [13], [14], [15]. Transcription factors such as Abf1 and Reb1 are considered activators of RiBi genes; frequently observed in promoters of those genes [16].

Under a stressing growth condition, in *S. cerevisiae*, TOR complex 1 senses the stress and, as a consequence the kinase Sch9 remains inactive. Dot6 and Tod6 are hypophosphorylated and active, then they recruit the histone deacetylase RPD3L to change the chromatin conformation to a closed state. In this condition, The activator Sfp1 is released to the cytoplasm from the nucleus, and the transcriptional activating effect over the ribosome biogenesis genes is abolished. Under a no stressing condition, TOR complex 1 activates the transcription factor Sfp1 and the kinase Sch9, Dot6 and Tod6 are hyperphosphorylated and inactive, then they are unable to recruit the RPD3L complex and the chromatin conformation remains open. Sfp1 is allocated to nucleus and activates the transcription of ribosome biogenesis genes. Additional to Spf1, there are other reported transcriptional activators as we pointed out above [12], [14], [15], [17], [18]. Besides RiBi genes, there is a set of genes that respond specifically to the nitrosative stress, some of them are the flavohemoglobin encoding gene *YHB1* (also known as the main scavenger of nitric oxide) [19], and the sulfite transporter encoding gene *SSU1*. The

zinc finger transcription factor Fzf1 is involved in the up-regulation of this gene set that responds to nitrosative stress [20].

Linde et. al. [21] observed that *C. glabrata* and *S. cerevisiae* have very different transcriptional responses to a nitrosative stress caused by GNSO in in vitro experiments. Interestingly, some of the GO categories of genes that were induced in response to nitrosative stress in *C. glabrata* are involved in ribosome biogenesis (RiBi genes), e. g. RNA polymerase III, maturation of SSU-rRNA, maturation of LSU-rRNA, endonucleolytic cleavage of SSU-rRNA, LSU-rRNA assembly and rRNA processing. They also tested the alkaline stress response in the pathogenic yeast by shifting the pH from acid (pH4) to alkaline (pH8) in minimal medium, and they observed that up-regulated genes are enriched in GO terms such as: glucose catabolism, glycogen metabolism and catabolism, and oxidation-reduction process. In addition, RiBi genes were repressed under alkaline stress. This profile is similar to that described in *S. cerevisiae* [22], [23].

Because to the interesting opposite direction of Ribi genes regulation observed in these two yeast, we decided to search for enriched motifs in the promoter sequences of the *C. glabrata* genes induced by the nitrosative stress in order to known the candidate trasncriptional factors involved in the regulatory orchestration triggered by this stress. We found enriched the PAC motif which is recognised by RiBi genes repressors Dot6 and Tod6, and the RRPE DNA motifs which is recognized by RiBi genes transcriptional activators on promoters of the induced genes by nitrosative stress response. We also found an enriched motif very similar to the MluI-box regulatory element which is recognized by the Mbp1 transcription factor who up-regulates DNA synthesis genes [24]. Gene set enrichment analysis, we performed in this work, suggests that *C. glabrata* apparently isn't under a stress condition that resembles to an oxidative or nitrosative stress, except for the significant over-expression of the gene *YHB1* that encodes for the first scavenger of nitric oxide, a flavohemoglobin with nitric oxide oxidoreductase activity [19].

In this work we proposed an early hypothesis which could explain, at certain limits, the mechanism of nitrosative stress response in *C. glabrata*. We based this hypothesis on analysis of previous RNA-seq data and on motif and gene set enrichment analyses we performed in this work. We think over-expression of *YHB1* gene is enough to rescue *C. glabrata* from nitrosative stress. And probably RRPE and MluI cell-cylce box DNA motifs are important *in cis* elements which mediate the up-regulation of the genes by the stimulus of 0.6mM GSNO. In this scenario we think that Dot6 or Tod6 repression is abolished. We dare to propose that GSNO or nitrate is a molecule that, by a molecular mechanism we do not know, elicits the transcription of RiBi genes.

2 Materials and methods

2.1 Getting and cleaning data

We downloaded the RNA-seq data set from NCBI SRA database (GEO: GSE61606) [21]. We tested the reads quality with FastQC [25] and, trimmed and cleaning with Trimmomatic [26]. We removed leading and trailing bases with a phred33 score lower to 28. Reads were scanned with a sliding window of 4 bases of length and we discarded reads with a subsequence of 4 bases with an average phred33 score lower than 26. After trimming, we dropped reads with a length lower than 36 bases. We searched and decoupled adapters using the Fasta files that defines the common adapters used in paired-end and single-end sequencing. We used all reads that passed the quality control in the subsequent mapping process.

2.2 Reads mapping and counting

We mapped the reads on the *Candida glabrata* reference genome (ASM254v2) using the reads mapper hisat2 with the default parameters [27]. Output SAM files were transformed to BAM files and sorted with samtools package [28]. Mapped reads to reference genome were allocated to *C. glabrata* gene annotations (ASM254v2) and counted with featureCounts function from Rsubreads R package [29].

2.3 Differential expressed gene analysis

We used the counts data frame to perform a differential expressed gene analysis using the edgeR package. We took into account treatment conditions and RNA-Seq library type (paired-end or single-end) in the design of the model matrix. We filter out annotated genes from counts data frame having zero or few allocated reads using filterByExpr function of edgeR package [30], [31]. Counts were normalized, first within lane, using the loess normalization with the upper-quartile method of withinLaneNormalization function taking into account the GC percentage bias for every annotated gene, and second between lanes using betweenLaneNormalization function; both functions we used are part of the EDASeq R package [32].

We performed differential expression analysis with the utilities of edgeR package. After data normalization, library size normalization factors were calculated for each sample, we calculated the common, tagwise and trended dispersion parameters. We fit the parameters of the negative binomial generalized linear model using maximum quasi-likelihood given the data to perform a genewise statistical test given the GSNO vs. YPD or alkaline vs. acid contrast. We took into account the library type in the model design. From these statistical test we selected those genes that did not significantly change the expression between samples as negative controls for every contrast analysis to perform a normalization step where we removed the unwanted variation using the RUVg function of RUVSeq package [33]. We set the number-of-factors parameter (k) equals 1.

We added the normalization factors we obtained from RUVg process to the design of the model matrix, and performed the differential expressed gene analysis again. We recalculate the library size and the library size normalization factors, we estimated the dispersion parameters and fitted the parameters of the negative binomial generalized linear model using the maximum quasi-likelihood given these data to perform the statistical test. We adjusted the p-values using the Bonferroni method.

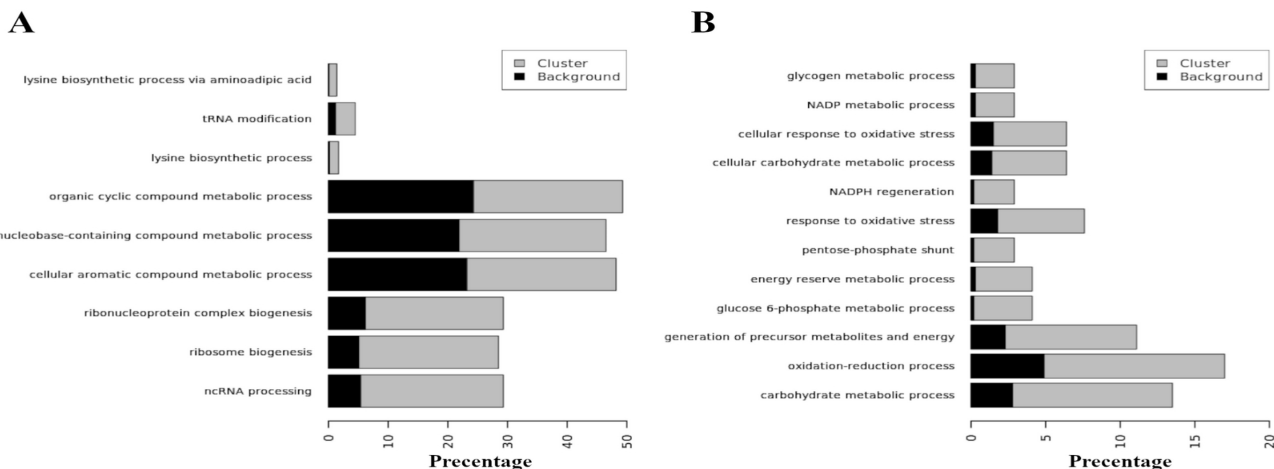

Fig. 1. GO term enrichment analysis. We plotted the results in barplots. gray bars represent the percentage of genes related to that GO term that are present in the sample cluster genes. Black bars measures the percentage of genes related to that GO term that are present in the total annotated genes of *C. glabrata*. We show in (A) results from GSNO vs YPD contrast analysis, and in (B) the results from pH8 vs pH4 contrast analysis. Significance of enrichment analysis was tested with hypergeometric distribution. We show some of the results with significant enrichment (adjusted p-value < 0.01).

2.4 K-mer enrichment analysis

We extracted the promoter regions of tested genes of *Candida glabrata* in the differential expressed gene analysis and limited them to the intergenic region or 1500bp upstream of the gene if the intergenic region overextends this size. We masked promoter low complex subsequences with dust software using default parameters and dropped duplicated promoters with purge [34]. We sorted the promoters from the most significantly expressed gene to the most significantly repressed gene based on the differential expressed gene analysis given either GSNO vs. YPD or alkaline vs. acid contrast. To achieve this sorting we calculated a gene expression estimate which is the negative logarithm of p-value multiplied by -1 if the logFC is negative and by +1 if logFC is positive (-log(p-value)). We used Sylamer software to perform the promoters k-mer enrichment statistical test to measure the significance using the hypergeometric distribution along the sorted promoter sequences using a sliding window of determined size. We compute all posible k-mers of length 7 nucleotides to test its enrichment and we used a window size of 300 promoters to perform this test [35].

2.5 GO Terms enrichment analysis

We selected the over-expressed genes set from both contrast (GSNO vs. YPD or alkaline vs acid) in the differential expressed gene analysis. In order to do this, we selected those genes that showed a logFC higher or equal to 1.5 and an adjusted p-value lower or equal 0.05. We used the systematic gene names of *Candida glabrata* to perform the GO term enrichment analysis in those dataset with the GO term finder online resource from www.candidagenome.org/cgi-bin/GO/goTermFinder [36]. We selected all annotated genes of *C. glabrata* as the background data set and the cellular process ontology to search in.

2.6 DNA motif discovery

We selected two data sets of promoter sequences of up-regulated genes in nitrosative stress and in alkaline stress selecting those genes with lgFC > 0 or logFC >= 1.5 and the adjusted p-value <= 0.05. Promoter low complexity regions were masked with dust program using default parameters. We perform the DNA motif discovery analysis using the online resource DREME from MEME suit (meme-suit.org) [37], [38]. To associate the DNA motif enrichment found in promoters to gene expression function, we calculated the expression estimates values as the negative logarithm of the p-value of the tested logFC in DEG analysis multiplied by -1 if the logFC is negative and by +1 if logFC has a positive value (-log(p-value). Then, we selected two population of genes, the first have no DNA motifs identified on its promoter (background population), and the second population has at least one copy of the tested DNA motifs on its promoter (test population). DNA motif counts on promoter sequences were done with countPattern function of Biostrings R package [39]; we searched in both strands for every discovered motif. Finally, we performed wilcoxon rank-sum test over those population asking if test population is greater than background. We plotted the negative logarithm of the wilcoxon rank-sum test p-values in a horizontal barplot. The bigger negative logarithm of p-value is, the more significant population shift is between the two populations. We performed an additional test. We randomly selected the expression estimate values (-log(p-value)) of 1000 samples having the same size than the test population from the entire dataset, and we plotted those gene expression estimates in cumulative frequency plots together with gene expression estimate values for background and test population.

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3 Results

3.1 Differential expressed genes

We observed that counts from some RNA-Seq libraries showed huge variation between samples (Supplementary Fig. 1A) and after within and between lane normalization, variation from the counts of RNA-Seq samples was removed (Supplementary Fig. 1B). Multidimensional plot shows that almost all samples of the same group tend to be closer to each other. Some samples are separated from the main group, and this may be due to technical variation sources (Supplementary Fig. 2). We observed a common dispersion lower than 0.2 of biological coefficient of variation in dataset where data was normalized using no differential expressed genes in the DEG test given GSNO vs. YPD contrast (Supplementary Fig. 3A), and alkaline vs. acid contrast analysis (Supplementary Fig. 3C).

We analyzed 5104 genes of *C. glabrata*, of which 1191 showed a significant differential expression under nitrosative stress; 332 out of 1191 are over expressed (logFC >= 1.5), and 112 are repressed (logFC <= -1.5) (Supplementary Fig. 3B). Under alkaline stress, we observed that 1117 genes showed significant differential expression profile; 137 out of 1117 genes were over expressed (logFC >= 1.5) and 166 genes were repressed (logFC <= -1.5) (Supplementary Fig. 3D).

We found that up-regulated genes in response to nitrosative stress are enriched in gene onthology terms related to ribosome biogenesis as was reported in [21]. We observed other GO terms like metabolic process of organic cyclic compounds and biosynthetic process of lysine (Fig. 1A). In alkaline stress, we found enriched GO term related to carbohydrate and energy reserve metabolism as well as to the response to oxidative stress (Fig. 1B).

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3.2 Promoters of the induced genes in the nitrosative stress are mainly enriched in PAC and RRPE DNA binding motifs

Under nitrosative stress elicited by 0.6mM GSNO in YPD, a mesurable proportion of *C. glabrata* genes that respond to this stimuli are involved in ribosome biogenesis processes, like expression of ribosomal proteins and proteins involved in ribosome maturation. This observation is very interesting because it contrasts to the transcriptional response that is observed in different stresses including the nitrosative that *Saccharomyces cerevisiae* faces [8], [21]. And, also the nitrosative stress response of *Candida glabrata* is different to that observed in *Saccharomyces cerevisiae* and *Candida albicans* [40]. With these antecedents in mind, we wanted to investigate the enrichment of putative in cis regulatory elements using a k-mer enrichment statistical test (Sylamer) over the promoter sequences of sorted genes based on the expression observed in each contrast analysis.

We show the top 15 enriched k-mers in promoter sequences of over-expressed genes in the GSNO vs. YPD contrast analysis (Fig. 2A), and in alkaline vs. acid contrast (Fig. 2B). We observed a highly and significantly enrichment of these 15 k-mers in the promoters of induced genes in nitrosative stress compared to the repressed genes in the same stress. In addition, we observed that 11 out of these 15 k-mers could be assembled into a bigger motif which is very similar to the PAC DNA binding motif [11], [41], [42] (Fig. 2E & Table 1). We also found a k-mer very similar to the reported RRPE DNA binding motif (GAAAATT), and two k-mers very similar to Skn7 motif [43] (Fig. 2A). Under alkaline stress, we found only two k-mers somewhat enriched but significant (TATTTAC & GCGATAT). Additionally, we observed conspicuous gray lines that tend to decrease toward the negative values of the y axis (Fig. 2B).

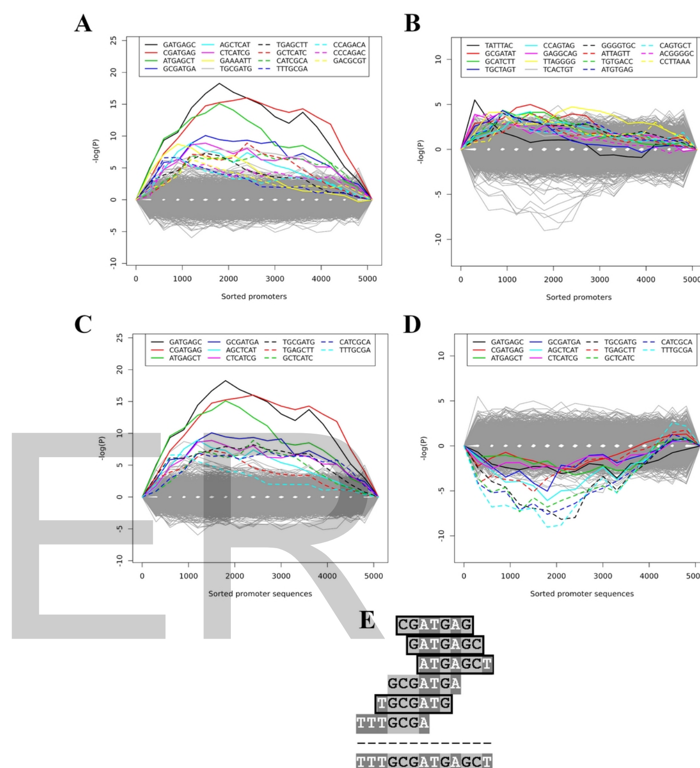

Fig. 2. Kmer enrichment analysis. Enrichment was tested with Sylamer software. Promoter sequences were sorted from the most significantly expressed gene to the most significantly repressed gene based on a differential expressed gene analysis in (A and C) nitrosative stress (GSNO vs YPD contrast), and in (B and D) alkaline stress (pH8 vs pH4 contrast). In (A and B) we plotted the top 15 kmers enriched in the up-regulated gene set. And in (C and D) we plotted only the kamers which can be assembled into Dot6 and Tod6 putative DNA binding motif. In (E) we show the assembling of some of the most enriched k-mers found in sylamer analysis over promoters sequences of the sorted genes based on GSNO vs YPD contrast. Boxes with black borderlines highlights k-mers whose reverse complement is also present in the most enriched k-mers set.

These conspicuous gray lines caught our attention because the k-mers they represent are depleted on the promoters of genes that were induced upon alkaline stress. Shifting pH from acid to alkaline causes a stress in *C. glabrata* so, we should expect, as is observed in *Saccharomyces cerevisiae*, that ribosome biogenesis genes are repressed in this condition, thus promoter sequences of the induced genes under alkaline stress should be depleted on k-mers similar to PAC DNA motifs. With this in mind we speculate that those gray lines with

minimum peak in the negative values of the y axis represent the k-mers that are similar to the PAC DNA motif. To test this, we plotted those k-mers with colored lines in both k-mer enrichment analysis where promoters were sorted based on the transcriptional expression profile given GSNO vs. YPD contrast analysis (Fig. 2C) or alkaline vs. acid contrast analysis (Fig. 2D). We observed that a measurable proportion of conspicuous gray lines were colored (Fig. 2D). That means up-regulated genes under alkaline stress are depleted of PAC DNA motifs.

Table 1. Dot6 and Tod6 DNA binding motifs.

TF	Reference	DNA binding site [a]
Tod6	Zhu et al., 2009	aDc**gCGATGAGSt**xHxV
Tod6	Zhao et al., 2009	**cGATG**
Tod6	Badis et al., 2008	**CGATG**SSc
Dot6	Zhao et al., 2009	**gcGATGag**
Dot6	Zhu et. al., 2009	axgax**gCGATGAGSt**gH
Dot6	Badis et al., 2008	**cGATG**

a. Bold section of the motif is highly similar to the motif found in C. glabarata promoters of genes induced in response to nitrosative stress.
b. MITOMI: mechanically induced trapping of molecular interaction, PBM: protein binding microarrays: Source www.yeastract.com.

These results support what Linde et al. [21] reported. They found that induced genes in nitrosative stress are involved in the ribosome biogenesis process such as: rRNA subunits assembly and maturation, rRNA processing and RNA polimerase III activity, it is expected to found the promoters of induced genes, as response to this stress, to be enriched of PAC and RRPE DNA motifs.

3.3 Several DNA motifs are associated to gene transcriptional activation under nitrosative or alkaline stress

In order to know what are the elements who orchestrate the gene transcriptional regulation triggered by nitrosative or alkaline stress, we performed a motif enrichment analysis using a different bioinformatic approach. We used the discriminative regular expression motif elicitation (DREME) online resource (meme-suit.org) as is described in material and methods section. Using the promoters sequences of the up-regulated genes (logFC > 0 & adjusted p-value <= 0.05) under nitrosative stress we discovered 24 DNA motifs; and under alkaline stress we found 21 motifs. We tested, with wilcoxon rank-sum test, if the gene population whose promoter sequence contains at least one copy of the tested motif (test population) has a greater transcriptional expression estimate (-log(p-value), see material and methods section) than the gene population with no DNA motif identified in its promoter (background population).

We found six significant DNA motifs (Fig. 3A), three of them are very similar to the PAC DNA motif (GCGATGMS, GAWGAGC and the reverse complement of CATCGM). We also found a motif similar to RRPE motif (reverse complement AAWTTTTC). With this result, we confirmed the enrichment of PAC and RRPE motifs in promoters of genes that were up-regulated under nitrosative stress. Additional to these motifs, we found the motif ACGCGTMA which is very similar to the reported DNA motif of Mbp1 transcriptional factor involved in regulating cell cycle progression (ACGCGT) [24], [44], [45]. We found that the median of the trasncriptional expression estimates of genes whose promoters contain at least one copy of the tested motif (blue line in Fig. 3B-G) conspicuously shifts from the observed median from the genes whose promoters contain no tested DNA motif (black line in Fig. 3B-G). Genes which are enriched with Mbp1 DNA motifs are also involved in process such as, DNA synthesis, mitosis and cell cycle (supplementary file 1).

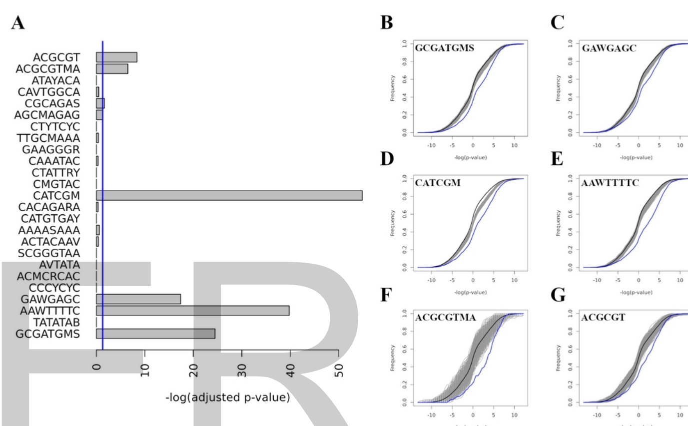

Fig. 3. DNA motifs discovery associated to gene regulation in nitrosative stress response. We plotted in (A) the negative logarithm of the adjusted p-values obtained from wilcoxon sum-rank test over two populations of gene expression estimates values (-log(p-value) of tested logFC in DEG analysis, given GSNO vs YPD contrast) for every tested DNA motif found using DREME. The first population corresponds to those genes who have no DNA motif in its promoter (background), and the second population of genes has one or more copies of the tested DNA motif (test). Blue line represents the significance threshold (-log(0.05)). In (B-G) We plotted the cumulative frequency of gene expression estimates values (-log(p-value)) for both population we defined above. The black line shows the population who has no DNA motifs in its promoter (background), and the blue line shows to the test population. Gray lines are the cumulative frequency of (-log(p-value)) from 1000 samples randomly selected with size equals to the test population from the entire population of gene expression estimates values.

Under alkaline stress, twenty-one DNA motifs were discovered (Fig. 4A), four of them are associated to gene transcriptional activation (they have significant wilcoxon rank-sum test p-values). No one is similar to the significantly enriched motifs found using sylamer approach. Three of them were no similar to reported transcriptional factors in *S. cerevisiae* (Fig. 4B, D-E). Except for the CCCTTY DNA motif which is similar to the motif who is recognized by Nrg1, Msn4 and Msn2 transcriptional factors involved in glucose repression and stress response (Fig. 4C) [42], [46], [47]. We observed that data distribution of gene expression estimates (-log(p-value))

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for the test gene population (blue line) is not equal to the background gene population (black line) (Fig. 4B-E). The data distribution of 1000 randomly selected samples (gray lines in Fig. 4B-E) are also slightly different to the distribution of the expression estimates for the test gene population.

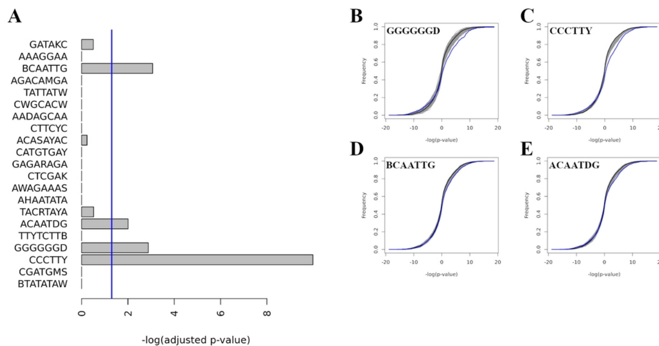

Fig. 4. DNA motif discovery associated to gene regulation in response to alkaline stress. We plotted in (A) the negative logarithm of the adjusted p-values obtained from wilcoxon sum-rank test over two populations of gene expression estimates values (-log(p-value) of tested logFC in DEG analysis given pH8 vs pH4 contrast) for every tested DNA motif found using DREME. The first population corresponds to those genes who have no DNA motif in its promoter (background population), and the second population of genes has one or more copies of the tested DNA motif (test population). Blue line represents the significance threshold (-log(0.05)). In (B-E) We plotted the cumulative frequency of gene expression estimates values (-log(p-value)) for both population we defined above. The black line shows the background population, who has no DNA motifs in its promoter, and the blue line shows to the test population. Gray lines are the cumulative frequency of (-log(p-value)) from 1000 samples randomly selected with size equals to the test population from the entire population of gene expression estimates values.

We performed a similar analysis using DREME online resource, but only with the promoter sequences of over-expressed genes under nitrosative or alkaline stress, that mean those genes with logFC bigger or equals to 1.5 and adjusted p-value lower or equals to 0.05. Under nitrosative stress condition, we discovered fourteen DNA motifs, but three of them have a significant wilcoxon rank-sum test p-values, comparing the test gene population with the background gene population (Supplementary Fig. 4A). Distribution of test and background populations are different, and the median of the expression estimates of test gene population noticeably changes compared to the median from background gene population (Supplementary Fig. 4B-D). DNA motifs found in this analysis are very similar to PAC and RRPE DNA motifs. Mbp1 DNA motif was not found in this analysis. Under alkaline stress we found 8 DNA motifs, and three of them showed significant wilcoxon rank-sum test p-values (Supplementary Fig. 5A). Distribution of the data from test and background gene populations is not equal (Supplementary Fig. 5B-D). These three DNA motifs we discovery in this analysis (CYCCCCY, CAATTSG and ACAATGG), are very similar to DNA motifs we found in the previous DREME analysis in alkaline stress response.

3.4 *Candida glabrata* transcriptional profile does not resemble a stress condition

Is *C. glabrata* under a stress condition, or isn't it when it grows on YPD plus 0.6mM GSNO? That's the question. In order to answer this question, we performed a gene set enrichment analysis using *C. glabrata* gene sets whose orthologous in *Saccharomyces cerevisiae* responds to one of several stress environments. Previously, we showed that ribosome biogenesis genes were over expressed under a nitrosative stress in *C. glabrata*, that was totally in contrast to what was observed in the published transcriptional profile of *S. cerevisiae* [21]. We tested the gene set enrichment using the ribosome biogenesis otrhologous genes of *C. glabrata* (we found 188 genes) and confirmed that these genes are over enriched in the up-regulated gene set during a nitrosative stress (Fig. 5A), and over enriched in the down-regulated gene set under pH shift stress condition (Fig. 5B). Also, it is reported that nitrosative stress and alkalinized medium also elicit oxidative stress pathways in *S. cerevisiae* [22], thus we evaluated the gene set enrichment using the *C. glabrata* genes whose orthologs in *S. cerevisiae* responds to oxidative stress. We can observe in (Fig. 5C) that these genes tend to be slightly enriched toward the down regulated genes under nitrosative stress, although the enrichment is not statistically significant. However they are enriched in the up regulated gene set under alkaline stress (Fig. 5D). In addition we also tested the genes which encode enzymes with catalytic activity involved in counteract oxidative damage such as: catalases, peroxidases, and thiorredoxin reductase. We found that these genes are slightly enriched toward down regulated genes under nitrosative stres (Fig. 5E), but highly enriched toward the up regulated genes under pH shift stress condition (Fig. 5F).

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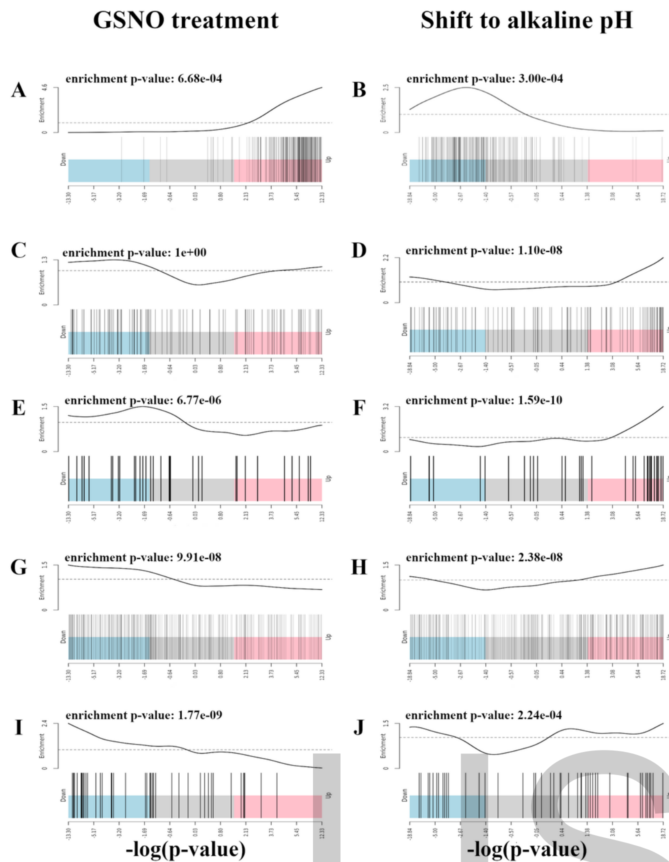

Fig. 5. Barcode plots. We plotted the position of every gen of different gene sets as vertical black lines along the total *Candida glabrata* annotated genes sorted from the most significant expressed gene to the most significant repressed gene. The blue and red zones represent the down- and up-regluated genes respectively. Upside this barcode plot is the worm plot, and it represents the enrichment fold of the gene set along the sorted *C. glabrata* genes. We analized several gene sets of *C. glabrata* whose orthologs in *S. cerevisiae* corresponds to: (A & B) ribosome biogenesis genes, (C & D) oxidative response genes, (E & F) genes that encode enzymes whose catalytic activity defends against cell oxidative damage, (G & H) genes that responds to pH stress, and (I & J) genes that responds specifically to alkaline stress.

We selected genes of *C. glabrata* whose orthologs in *S. cerevisiae* responds to any pH shift (Fig. 5G & 5H) and only those genes that responds only to alkaline pH (Fig. 5I & 5J). In both cases we observed similar behaviors: significant enrichment toward down regulated genes under nitrosative stress, and under alkaline stress we observed slightly but significant enrichment toward the up regulated genes.

Based on gene set analysis results, we think *C. glabrata* seems to be under a stress condition when the pH shifts from acid to alkaline, but it seems not to be in such a stress condition when it grows in YPD plus 0.6mM GSNO (a nitrosative stress). In spite of that transcriptional profile of *C. glabrata* observed during nitrosative stimuli suggests a no stressing environment, we found over expressed the flavohemoglobin gene which has nitric oxide oxidoreductase activity (logFC: 5.61; adjusted p-value: 9.93e-08). *YHB1* is a well known and conserved gene; fungi research community call it the first scavenger of nitric oxide that protects yeast and other fungi from nitrosative stress [19], [20]. In addition, we observed that *C. glabrata* genes whose orthologs in *S. cerevisiae* responses directly to nitrosative stress are enriched toward the up regulated genes when the pathogen yeast is facing that stress condition, although logFC is not statistically significant (Supplementary Fig. 6A). *YHB1* trasncriptional expression does not significantly change when pH shifts from acid to alkaline, and there is not a gene set enrichment (Supplementary Fig. 6B).

4 Discussion and Conclusion

Candida glabrata is a pathogenic yeast which has the ability to grow and survive inside the macrophage. This yeast is engulfed by the macrophage phagosome, once inside, it is able to evade the immune system and phagocytosis. *C. glabrata* has the ability to change the pH from acid to alkaline into the lysosome in order to inactivate lytic enzymes of the phagosome [1], [2]. Nitric oxide synthase gene (iNOS) of the macrophage is involved in the production of nitric oxide and thionitrosogluthatione (GSNO). This chemical compound is an excellent nitric oxide donor that causes nitrosative stress [48].

Little is known about how *C. glabrata* responds to a nitrosative stress. In contrast, in the close relative and well-studied budding yeast *S. cerevisiae*, it is known that when this yeast faces one or several stresses, including nitrosative stress, there is a global transcriptional profile change, and one of the most conspicuous changes is the repression of the ribosome biogenesis genes [8], [9], [10]. The genes that belong to this group have in cis-regulatory elements in its promoter (PAC & RRPE DNA motif). PAC motif is recognized by transcription factors Dot6 and Tod6; they are the repressors of these genes. Under a non-stressing condition these repressors are dephosphorilated and inactive, thus ribosome biogenesis genes are not repressed. Spf1 is an activator of the transcription of those genes who recognizes the RRPE DNA motif, and in this condition this transcription factor binds at the promoters of ribosome biogenesis genes [14], [15]. In addition, it has been proposed that general regulatory factors such as, Abf1 and Reb1 recognize upstream activating sequences and activate the transcription of those genes [16]. Under a stressing condition, Dot6 and Tod6 are hypophosphorilated and active. When these transcription factors are active, they recruit the histone deacetylase (HDAC) RPD3L complex and close the chromatin by the activity of this HDAC [12], [14], [15], [17], [18]. There is another repressor called Stb3 who recognizes the RRPE DNA motif which together with Dot6 and Tod6 repress ribosome biogenesis genes [49], [16]. Under this stressing condition, Spf1 is relocated to cytoplasm and its activating effect is abolished [14], [15].

In *S. cerevisiae*, this scenario of transcriptional regulation leads to repress ribosome biogenesis genes under a stress condition and leads to up-regulate RiBi genes under non-stressing condition. However, in *C. glabrata* we observed that this scenario is quite different; under nitrosative stress, caused by addition of 0.6mM of GSNO to YPD medium, this pathogenic yeast up-regulates ribosome biogenesis genes instead of represses them. Results from gene set enrichment analyses suggest that *C. glabrata* is not under a stress condition. Oxidative stress is associated to nitrosative stress because pathways that are turned on under oxidative stress are also active under

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nitrosative stress, and nitric oxide promotes the induction of ROS from Lipoperoxidation [50], [51]. However addition of 0.6mM of GSNO did not up-regulate genes involved in oxidative stress response neither genes that encode enzymes that protects cell from oxidative damage. Nevertheless, the flavohemoglobin *YHB1* which transforms nitric oxide to nitrite was conspicuously expressed, and the genes of *C. glabrata* whose orthologs in *S. cerevisiae* responds to nitrosative stress tend to be enriched toward the up-regulated genes in response to nitrosative stress, although its logFC is not statistically significant. We think that in *C. glabrata* the flavohemoglobin, encoded by *YHB1* gene, contends against nitrosative stress caused by 0.6mM GSNO and we think it is enough to mitigate this stress making it unnecessary to up-regulate genes whose orthologs in *S. cerevisiae* respond to oxidative and nitrosative stress.

Candida glabrata is not under a stress condition when it gorws in YPD medium, it can efficiently grow in this medium, that means, ribosome biogenesis genes are transcriptionally active. Results from gene set enrichment analysis suggest *C. glabrata* is apparently not under a stress condition in YPD + 0.6mM GSNO because transcriptional profiles do not resemble to a stressing scenario, except for the over-expression of *YHB1* gene. If both growth conditions do not represent a stress condition we should expect similar expression levels of ribosome biogenesis genes, however we observed in this work as well as in Linde et al. [21] work that ribosome biogenesis genes are over-expressed when yeasts were treated with 0.6mM GSNO. This observation suggests that GSNO or nitrite molecules could trigger the over expression of ribosome biogenesis genes.

When *C. glabrata* grew on a minimal medium upon a pH shift from acid to alkaline, we observed that transcriptional profiles and GO term enrichment analysis resembles to a stress condition. It is known that medium alkalinization causes lose of the activity of plasmatic membrane nutrient transporters, and also this condition is related to oxidative stress because under alkaline stress *S. cerevisiae* accumulates reactive oxygen species. Some other GO term which are enriched on the up-regulated genes in response to alkaline stress in the baker's yeast, are glycogen metabolism and biosynthesis, oxidation-reduction process and monosaccharide catabolic process [22]. And those GO terms are also enriched in the up-regulated genes in response to this stress in *C. glabrata*, in addition to the energy reserve metabolic process GO term. We also observed that the set of genes whose orthologs in *S. cerevisiae* respond to pH stress are significantly enriched toward the up-regulated genes under alkaline stress in *C. glabrata*. So we think that *C. glabrata* responds to alkaline stress in a similar way as *S. cerevisiae* does.

4.1 An early bioinformatic approach to the model of nitrostives stress response in *Candida glabrata*

Candida glabrata is engulfed by phagocytosis, once inside macrophage, we think, it counteracts nitrosative stress caused by thio-nitrosogluthatione (GSNO) generated by the macrophage and the alkaline stress caused by the increment of the pH that this yeast give rise to inhibit the lytic activity of the phagosome enzymes. Under alkaline stress, the macrophage's malevolent guest is exposed to face nutrient unavailability such as monosaccharides like glucose. In this scenario, the yeast utilizes the energy reserves such as glycogen to obtain carbon and energy sources. Meanwhile, the flavohemoglobin *YHB1* gene is catalyzing the conversion of nitric oxide donated from GSNO to nitrite in order to inhibit or reduce the damage that this reactive nitrogen species can cause to the cell. And Dot6 and Tod6 could remain inactive and RiBi genes repression could be overturned.

We think that if GSNO or nitrite molecules are present whether inside the yeast cell or in macrophage's cytoplasm, it trigger the transcription of ribosome biogenesis genes faster than if these molecules are not present. At this stage we have no evidences to know if the signal of the gene transcription induction is direct or it passes through TOR complex 1. We do not have evidences to propose the elements that transduce the signal, neither the transcriptional factors involved in the induction of ribosome biogenesis genes, but we think they could be Spf1, Abf1 or Reb1 transcription factors in C. galbrata based on the homology to *S. cerevisiae* and the RRPE motif enrichment on promoters of the up-regulated genes (Fig. 6). There was also found a DNA motif similar to the motif of Mbp1 transcription factor which associates to Swi6 enriched in promoter sequences of a fraction of the up-regulated genes (60 genes) in response to 0.6mM GSNO. This fraction of up-regulated genes are enriched in cell cycle process and DNA synthesis (Supplementary file 1). This finding suggest that GSNO or nitrates trigger also the cell cycle process. The relevance of this hypothesis relies in, that this early model could represent a therapeutic target to design drugs or develop strategies to counteract *C. glabrata* infections in immuno-compromised patients.

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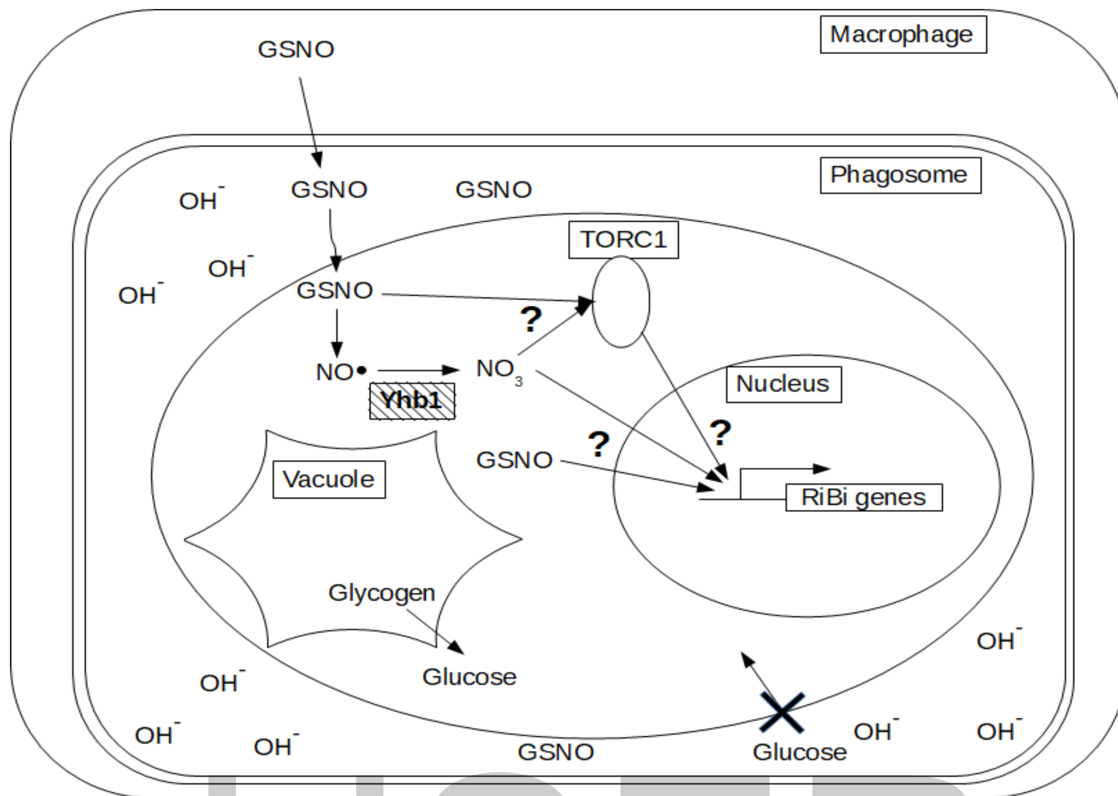

Fig. 6. Early model to nitrosative stress in *Candida glabrata*. Here we depict what we think about how the pathogenic yeast *C. glabrata* responds to a nitrosative stress. GSNO, thionitrosogluthatione molecules. OH-, alkaline environment. RiBi genes, ribosome biogénesis genes. Yhb1, flavohemoglobin with nitric oxide oxidoreductase activity. TORC1, target of rapamycin complex 1. Question marks represent holes in the model.

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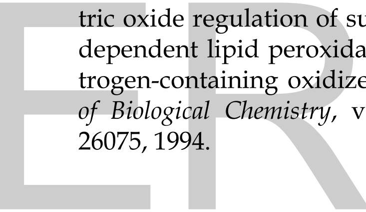

Supplementary material

Supplementary Fig. 1. 10.6084/m9.figshare.14156873.
Supplementary Fig. 2. 10.6084/m9.figshare.14156876.
Supplementary Fig. 3. 10.6084/m9.figshare.14156879.
Supplementary Fig. 4. 10.6084/m9.figshare.14156882.
Supplementary Fig. 5. 10.6084/m9.figshare.14156885.
Supplementary Fig. 6. 10.6084/m9.figshare.14156888.
Supplementary file 1. 10.6084/m9.figshare.14167055.