SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of a Fungus Specific Protein Phosphatase

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13:00 pm, 7th September 2020.

Introduction

Invasive candidiasis is a severe infection caused by fungi of the *Candida* genus. The most common species isolated from patients is *Candida albicans*. According to a study completed between 2008 and 2011, 885 out of 2329 clinical isolates contained *C. albicans*, although other *Candida* species - like *Candida glabrata* or *Candida parapsilosis* – were also widespread among pathogens causing nosocomial infections. *C. albicans* constitutes part of the normal human microbiome. This opportunistic pathogen persists in 65% of healthy individuals without causing any serious symptoms. However, if environmental conditions change, it can turn to a dangerous pathogenic fungus. It is known that candidiasis can be fatal in patients with a compromised immune system (by AIDS, autoimmune diseases, immunosuppressive therapy etc.). There is a high risk of infection among diabetics, cancer patients and the elderly as well as among people undergoing steroid and antibiotic therapy. Since the resistance against antifungal agents is steadily increasing, it has become necessary to develop new drugs and combined therapies that can effectively combat the overgrowth of *C. albicans*. Most of the currently used medicines target signaling pathways that is why we deem that a signal transduction therapy may be suitable for the treatment of *C. albicans* infections. One of the most common signaling mechanisms involves the post-synthetic modification of proteins by protein phosphorylation and dephosphorylation. Several known drugs act on protein kinases that catalyze the phosphorylation of key proteins. As all of these kinases originate from the same ancestor, they possess a common catalytic site that hampers the design of highly specific inhibitors. That is why it can be a promising approach, if we select a drug target from the family of protein phosphatases that catalyze the dephosphorylation reaction. Due to its quite complex convergent evolution, the family of protein phosphatases is more diverse, and includes several fungus specific members. One of the fungus specific Ser/Thr phosphatases is protein phosphatase Z1 which was first identified in the classical model organism *S. cerevisiae*. There is only one protein phosphatase Z gene in *C. albicans* which was named *CaPPZ1* and was shown to have important roles in several physiological processes. We suggest that the product of this gene, the CaPpz1 protein can be a promising antifungal drug target based on the following considerations:

It is known that CaPpz1 plays a role in virulence. Deletion of the *CaPPZ1* gene does not kill the fungal cells, but it delays cell adhesion and slows down the subsequent yeast-to-hypha

morphological transition. Consequently, inhibition of the CaPpz1 phosphatase can block the development of the invasive hyphal forms which are associated with virulence. According to our results *C. albicans* can compensate the lack of the phosphatase, thus the inhibition of the phosphatase would have only mild consequences. Therefore we think that in a combined therapy we have to interfere with another signaling pathway as well, in order to trigger more robust antifungal effect. Previously, it was demonstrated that CaPpz1 has a role in oxidative stress response. We suggest that the inhibition of the phosphatase in combination with a relatively mild oxidative stress treatment could form the bases of a specific antifungal therapy.

It is known that the CaCab3 protein acts as a physiological inhibitor of CaPpz1, but this universal essential protein cannot be considered as an optimal drug material. A low molecular mass organic inhibitor would be more desirable. Recently it has been reported that there are two CaPpz1 specific structural elements (L1-loop and a specific C-terminal helix) in the well conserved C-terminal catalytic domain, furthermore the highly variable N-terminal domain of the enzyme has a regulatory role. Based on these specific C- and N-terminal structural elements it looks reasonable that CaPpz1 specific inhibitors can be designed in the future.

To fulfill the above mentioned promising goals, first of all we have to establish strong theoretical bases of our starting hypothesis, and we have to extend our knowledge on the function and regulation of CaPpz1 phosphatase. Since most of the previous studies followed the logical guideline offered by the investigations of the classical yeast model, *S. cerevisiae*, it is conceivable that the virulence related functions of the filamentous *C. albicans* remained undetected. Therefore I used hypothesis-free global proteomic and transcriptomic approaches during my work to unravel new targets and functions of the phosphatase. My main research was focused on the combined effects of *CaPPZ1* deletion and oxidative stress treatment of the opportunistic pathogen.

Aims

Our main goal was to reveal new functions of the fungus specific CaPpz1 phosphatase under normal conditions and in oxidative stress induced by *tert*-butyl hydroperoxide (*t*BOOH). We aimed to investigate this scientific question by global proteomic and transcriptomic approaches. The *cappz1* phosphatase deletion mutant (KO) and the genetically matching QMY23 control (WT) strains of *C. albicans* have been available for these studies.

First, we planned to determine the long-term effects of the genetic mutation, i.e. the adaptation of the fungus to the lack of CaPpz1. We intended to use a combined phosphoproteomic and proteomic study supported by an RT-qPCR based transcriptomic experiment. We were ready to confirm experimentally the relevance of the yet unknown physiological functions which we hoped to identify during these investigations.

Second, we planned to investigate how the WT and KO cells reacted to a rapid oxidative stress treatment. To this end we wanted to introduce a proper experimental system which allows the comparative analysis of phosphatase deficiency, oxidative treatment and a combination of the two conditions together. For this we had to set the optimal culturing and treatment conditions so that they have well detectable effects, but do not cause drastic changes in cell growth, viability, vitality, and morphology. Since the QMY23 strain has not been investigated in these respects yet, we wanted to determine the behavior of both KO and WT strains under oxidative stress conditions. We planned to check the effectiveness of oxidative stress by measurements of antioxidant enzyme activity and determinations of glutathione concentrations. After testing and optimizing the experimental conditions we were going to perform the transcriptomic studies by two independent methods. As a preliminary experiment, we designed a DNA chip hybridization assay that shall be followed by the main next-generation RNA sequencing based experiments. The selected results of these would be confirmed by RT-qPCR.

From the above described experiments we expected to find new targets and functions for the CaPpz1 phosphatase. The planned global studies may provide new data on the interplay between the phosphatase and oxidative stress, and could contribute to the development of a novel antifungal therapy that is based on the combination of specific CaPpz1 inhibition and mild oxidative stress.

Materials and Methods

Culturing of *C. albicans* cells

In our experiments we used the *cappz1* phosphatase deletion mutant (KO) strain and the corresponding QMY23 control (WT) strain. Initial cultures were prepared from colonies grown on YPD medium at 37 °C for two days. Fresh overnight cultures were generated from them, and were diluted to identical optical densities. For proteomic studies, samples were taken from cultures at the end of the linear growing phase almost reaching log. For transcriptomic studies, cells were treated by *t*BOOH for 1 h at final concentration of 0.4 mM in the exponential phase.

Studies of biofilm formation

In vitro biofilm assays were performed in two different media: the common hydrogen-carbonate free RPMI-1640 and the more selective Spider medium. We determined the dry biofilm mass, the planktonic over not-adhered cell mass ratio as well as we performed crystal violet staining of the biofilm. Microscopic images of the cultures were taken at 40x magnification using an Olympus BD40 phase-contrast microscope with an attached digital camera.

Physiological tests

*t*BOOH treated and non-treated KO and WT cells were diluted, spread on YPD plates and their colony forming capacity was analyzed next day. Results were given as CFU/ml. Viability of the cells was analyzed with methylene blue staining and was visualized under phase-contrast microscope (*Euromex Holland*). Vitality of the cells was determined with Funga*Light*™ CFDA, AM/Propidium Iodide Yeast Vitality Kit. Cells were analyzed under Leica SP8 confocal microscope. Microscopic images were evaluated with ImageJ software. Morphology of the Funga*Light*™ stained cells was analyzed with flow cytometry in Novocyte 3000 *(Acea Biosciences, Inc.*) apparatus.

Enzyme activity assays and glutathione concentration measurements

C. albicans samples were mechanically disrupted for enzyme activity measurements. Protein concentration was determined by Bradford's method. Catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase activities were measured by photometry. For the measurement of glutathione contents, cells were suspended in 5% sulfosalicylic acid solution. Oxidized (GSSG) and reduced (GSH) glutathione concentrations were measured by the glutathione reductase-DTNB method.

Two-dimensional gel electrophoresis and mass spectrometry

Proteins were prepared from KO and WT cells for proteomic studies. Protein content was checked using Bradford reagent. Samples were cleaned using Ready-Prep 2D CleanUp Kit (*Bio-Rad*) and were immediately subjected to two-dimensional (2D) electrophoresis. We analyzed 3 biological replicates, i.e. 3 KO and 3 WT samples. First dimension was the isoelectric focusing using IPG (pH 4-7) strips. Second dimensional separation was performed by electrophoresis in 12% SDS-polyacrylamide gel. Gels were stained with Pro-Q Diamond dye which shows the phosphoproteome, then gels were stained with homemade RuBPS [Ruthenium(II) tris(bathophenanthroline disulfonate) tetrasodium salt] fluorescent dye which detects the whole proteome. Gels were scanned by Pharox FX Plus Molecular Imager machine.

Gel images were analyzed by Delta2D v4.4 (Decodon) software. The differences between the KO and WT samples were determined in three steps: First Pro-Q Diamond stained WT and KO gels were analyzed then gels were stained with RuBPS. Finally every spot was transferred to a mixed gel for spot excision. After gel image analysis, the software automatically calculated the mean normalized spot volume intensity changes between the two samples and the significant differences were assessed using Student's t-test. Those spots which showed significantly different ($p<0.05$) normalized spot volumes between KO and WT samples, were cut out from mix gel and subjected to in-gel digestion with trypsin. The tryptic peptides diffused out of the gel were aspired and dried. Protein identification was performed by mass spectrometry. Peptides were separated with Easy nLCII (Burker) nanoHPLC. The peptide mixture was loaded onto a Zorbax 300SB-C18 desalting column then it was separated with Zorbax 300SB-C18 analytical column. The peptides eluted from

the column were analyzed in a 4000 QTREP (*ABSciex*) mass spectrometer. The positive ion mode MS/MS spectra and the Information Dependent Acquisition (IDA) method were applied.

Protein identification was performed with ProteinPilot 4.5 software (*ABSciex*) based on MS/MS spectra. In questionable cases sequences were checked with NCBI non-redundant protein database using blastp program. All of our sequence annotations were confirmed by an independent data-mining of the CGD database in two steps.

Isolation of RNA

Lyophilized *C. albicans* cells were suspended in TriReagent. After mechanical disruption of the cells, chloroform was added to the samples to separate phases. This was followed by isopropanol addition to precipitate RNA. RNA was dissolved in nuclease free water.

RNA-sequencing

RNA-Seq libraries were generated from total RNA using RNA Sample preparation Kit (*Illumina*) according to manufacturer's protocol. Sequencing was performed with the Illumina NextSeq500 instrument. Approximately 18-20 million reads were generated for each sample. Reads were aligned to the reference genome (C_albicans_SC5314/ Assembly22 genome version). Tophat and Cufflinks bioinformatic tools were used for the genome mapping as well as for generating BAM files and RPKM (Reads Per Kilobase Million) values of A and B alleles. The RPKM values of the corresponding A and B alleles were merged and these values were used for the downstream analysis using StrandNGS software. Data were normalized by DESEQ1 algorithm. Log_2FC fold change values were calculated from normalized RNA-Seq data. Significance was determined using ANOVA test combined with Tukey post hoc test and Benjamini-Hochberg FDR (False Discovery Rate) test. Cluster v3.0 and Java TreeView v1.1.6r4 open source applications were used to generate heat maps. GO term analysis of up- and downregulated genes was carried out with the GO term Finder of CGD database according to function, component, and process GO terms. Only those enrichments were considered to be significant whose corrected p value was less than 0.05. RNA-Sequencing was performed by the UD-GenoMed Medical Genomic Technologies Ltd. in Medical and Health Science Center, University of Debrecen.

Reverse transcription and quantitative PCR (RT-qPCR)

In all cases cDNA was prepared from total RNA after DNase treatment. Primers used in quantitative PCR were designed with Primer3 v0.4.0 software and were checked with Oligo Analyser v1.0.2 program. 2x qPCRBIO SyGreen Mix Lo-ROX (*Nucleotest BIO*) master mix was used for quantitative real-time PCR. Measurements were carried out in Roche LightCycler® 480 II (*Roche*) instrument. The data were normalized to *ACT1* gene and relative gene expression of samples was calculated using ΔΔCP method. P values were determined by Student's t-test and by ANOVA test.

Results and Conclusions

Functions of CaPpz1 based on proteomic and phosphoproteomic studies

The phosphoproteomic/proteomic methodology was used for the investigation of the long-term consequences of CaPpz1 deficiency in *C. albicans*. These studies allowed us to reveal adaptation mechanism whereby the opportunistic pathogen can compensate the lack of CaPpz1. We found a total of 25 proteins, whose protein amount and/or phosphorylation level altered in the absence of CaPpz1. These proteins were categorized into 4 large groups: protein synthesis/degradation, morphological transitions, oxidative stress response, and metabolism.

12 out of 25 proteins take part in protein synthesis/degradation processes. 9 proteins are directly involved in translation; these include 1 translation initiation factor (Tif1), 3 translational elongation factors (Efb1, Eft1, and Cef3) and 5 ribosomal protein components (Rpp0, Rps7A, Rpl9B, Rpl20B, and Rps0A). In addition, 3 chaperones (Ssa4, Tsa1, and Crp3) that take part in protein folding and 2 proteins which are involved in protein degradation (Uba1 and Rad23) were identified. The amount of 7 proteins related to translation decreased, while the level of Uba1 (ubiquitin activating enzyme) that is promoting proteolysis increased in the absence of CaPpz1. These results suggest that protein synthesis slows down in the absence of the phosphatase. This observation is supported by the fact that in *S. cerevisiae* ScPpz1 has a negative impact on translation. In contrast, phosphorylation level of proteins was altered in a more diverse way in the phosphatase deletion mutant. Phosphorylation of Eft2 (elongation factor), Rad23 (DNA repair protein), and Rpp0 (ribosomal protein) increased, while phosphorylation of Rpl26 (ribosomal protein), Rps7A (ribosomal protein), Tif1 (elongation factor), and Uba1 (ubiquitin activating enzyme) decreased. Decreased levels of phosphorylation can be explained by the association of CaPpz1 with other regulators such as Sit4 phosphatase or MAP kinase with which Ppz1 interacts in an antagonistic or synergistic way, respectively. The Cef3 elongation factor was identified in two spots; while decreased protein levels were detected in both spots, protein phosphorylation was only detected in one spot. This means that the regulation of this protein is not mediated by dephosphorylation.

Based on our results we assume that Eft2 and Rpp0 may be the substrates of CaPpz1. The Eft2 translation elongation factor 2 is known to have a role in ribosome translocation during protein synthesis. According to our analysis several casein kinase 2 phosphorylation consensus sequences can be found in the *C. albicans* Rpp0 protein. Indeed, its yeast ortholog has been shown to be regulated by casein kinase, however this regulation is not essential during translation, but rather plays a role in osmoregulation. We built an interaction network for the 25 proteins which demonstrated that Eft2 as an elongation factor and Rpp0 as a ribosomal protein are important nodes of the network, suggesting that Ppz1 may regulate translation through these central proteins.

The role of CaPpz1 in oxidative stress has been previously published. Supporting this observation we identified 3 oxidative stress related proteins with altered protein content or phosphorylation level: Ure2 (glutathione peroxidase), Dug1-like (Cys-Gly metallopeptidase), and Tsa1 (thioreodoxin peroxidase). Our data suggest that the lack of CaPpz1 can affect the ratio of reduced and oxidized glutathione concentrations.

5 metabolic enzymes were also affected by CaPpz1 deficiency. Protein content of Idh1 (isocitrate dehydrogenase) and Tkl1 (transketolase) increased in KO cells. Phosphorylation of Aip1 (dehydrogenase), Guk1 (guanylate kinase), and Ham1 (inosine triphosphate pyrophosphatase) decreased in mutant cells. As I mentioned above, these protein dephosphorylation effects can be explained by the interplay between CaPpz1 and either the Sit4 phosphatase or the MAP kinase mediated pathways.

Finally we identified 3 proteins related to morphological transition: Ade12 (adenylsuccinate synthase), Bmh1 (14-3-3 scaffold protein), and Lia1-like (deoxyhypusine hydroxylase) protein. Protein content of Ade12 increased in the absence of CaPpz1, while protein level of Bmh1 and Lia1-like dropped in the mutant cells. These results are in good correlation with a previous published publication reporting that the expression of Ade12 is upregulated, while that of Bmh1 and Lia1-like proteins are downregulated during biofilm formation. Furthermore we found in a database search that 17 out of the 25 identified proteins may have a role in biofilm formation.

Collectively these data prompted us to investigate the effect of *CaPPZ1* deletion in biofilm formation experimentally. We found that KO mutant strain formed stronger biofilm in selective Spider medium. This is probably due to lower level of hypha formation and the higher proportion of yeast cells which can form a more compact biofilm structure in the KO cells. So we demonstrated that CaPpz1 phosphatase plays a role in biofilm formation, but

further investigations are needed to determine the physiological significance of this novel result.

Functions of CaPpz1 according to transcriptomic studies

We used a short *t*BOOH treatment to investigate the rapid response of the *C. albicans* to oxidative stress. Several methods (growth rate measurement, CFU counting, microscopy) were used to confirm that our experimental conditions were suitable for a global transcriptomic analysis. We have shown that a 1-hour treatment with a sublethal concentration of *t*BOOH slightly induces fungal hypha formation, reduces growth rate, but does not kill the cell, despite the fact that it inhibits their proliferation. In the case of KO samples, the fungistatic effect of *t*BOOH treatment was remarkable, supporting our idea of a positive interaction between phosphatase deletion and oxidative treatment. This interaction was confirmed by the elevation of the activity of antioxidant enzymes and a shift of reduced to oxidized glutathione concentration. Our biochemical results suggested that the redox status of cells was affected by phosphatase deficiency or oxidative treatment alone, but the two conditions amplified each other synergistically. It is important to note that the changes in oxidative enzyme activities obtained with the KO strain are new results, because they point out how CaPpz1 may affect the susceptibility of *C. albicans* to oxidative agents.

After a preliminary DNA chip experiment, we carried out an extensive RNA-sequencing (RNA-seq) study to determine the gene expression in *t*BOOH treated and non-treated WT and KO cells. The first results showed that the absence of phosphatase and the oxidative treatment alone have a slight effect on gene expression, but together they exhibited robust gene changes in *Candida*.

The RNA-seq data were subjected to a large-scale GO term analysis to determine which function-, localization-, or process-related genes were affected in the absence of phosphatase and under oxidative stress. By data analysis, five main gene categories were established, which are related to the terms of membrane transport, cell surface, oxidation-reduction, ribosomal proteins, and RNA metabolism. After a strict manual curation of data, heat maps were generated to show quantitative transcriptomic changes in all categories. Based on the heat maps and the available literature we selected 44 genes to validate their gene expression by RT-qPCR.

In the case of transport processes, we confirmed the increased expression of the *ENA2* sodium transporter gene in KO strain. It is known from the literature that *ppz1* KO *C. albicans* tolerates high salt stress well. With this experiment we proved one of the modes of action of phosphatase in salt homeostasis. Beside this we demonstrated the role of CaPpz1 in several additional transport mechanisms that have not been described yet. Expression of *PHO84* phosphate, *GIT1* glycerophosphoinositol and *HGT1* glucose transporters also increased in KO samples. The transcriptional regulation of *PHO84* by the phosphatase is especially interesting, because it acts as a potential regulator of *TORC1* (Target of Rapamycin complex 1) which regulates cell growth. Glycerophosphoinositol, taken up by Git1, serves as a source of inositol and phosphate in cells. *HGT1* is a high affinity glucose transporter whose expression increases in the presence of drugs. Our data suggest that CaPpz1 may be related to the regulation of drug resistance.

The role of CaPpz1 in oxidative stress response has been indicated by our biochemical experiments, which revealed that CaPpz1 can influence the oxidative state of the pathogenic fungus by altering the activity of oxidoreductase enzymes. According to our transcriptomic analysis, the expression of 3 genes encoding oxidase enzymes (*CFL2*, *FET31*, *SOD4*) increased significantly in KO strain, thus these genes may be regulated by CaPpz1. Altered expression of *CFL2* (oxidoreductase) and *FET31* (multicopper oxidase) suggest that CaPpz1 may be involved in iron transport modulation, as the product of these genes have iron reductase activity. *SOD4* that codes for a superoxide dismutase isoenzyme showed the most remarkable changes. Increased level of *SOD4* expression may help to survive the fungus in oxidative stress. This finding is in good agreement with biochemical assays, as the activity of SOD enzymes was elevated in KO cells. It is convincible that the activity of one of the SOD enzymes is regulated by CaPpz1 at the level of translation.

In conclusion, we demonstrated that phosphatase deficiency affects membrane transport and oxidation-reduction processes, which explains the characteristic phenotypes of the deletion mutant strain.

Next the effect of *t*BOOH treatment alone was examined. We compared the gene expression of the treated control strain with that of the non-treated control strain (WTt vs. WT) and found that the transcriptomic effect of mild oxidative stress was moderate.

The oxidative stress alone increased the expression of 4 genes involved in oxidation-reduction processes. The mRNA levels of *FET31* and *SOD4* increased in KO samples,

suggesting that oxidative stress and phosphatase deficiency have some common targets. In agreement with this, expression of *CFL4* and *SOD3* also increased in oxidative stress. Our results suggest that regulation of iron transport can be a common point in the signaling pathway of the CaPpz1 phosphatase and the *t*BOOH induced oxidative stress. Furthermore, the increased expression of two SOD isoenzymes is another mechanism which links phosphatase deficiency to the protection against oxidative stress.

As to the transport processes, the expression of *GAP1* amino acid transporter and *CDR1* ABC transporter genes increased upon *t*BOOH treatment. This shows that oxidative stress can modulate those transport processes that have a role in fungal survival mechanisms.

A total of 4 genes related to cell surface were identified, whose expression was suppressed by oxidative treatment alone: *CHT3* chitinase, *FGR41* adhesion protein, *PLB1* phospholipase B, and *CDC19* pyruvate kinase. The products of these genes act as virulence factors and cell surface markers.

Oxidative treatment alone slightly reduced the expression of genes encoding 5 cytosolic ribosomal proteins. These include 3 cytosolic ribosomal subunits (*RPL29*, *RPS3*, and *RPS8A*) as well as the ribosome-bound acidic ribosomal protein *RPP1B* and the translational elongation factor *CAM1*. Our results suggest that the diminished protein synthesis induced by oxidative stress can be the reason for the low colony forming capacity of the WTt cells.

Finally, our novel observation is the increased gene expression of RNA metabolism related genes under oxidative stress. We demonstrated that *t*BOOH stress upregulated the *BUD22* and *NSA2* genes involved in rRNA maturation as well as *SPB4* RNA helicase and C3_02750W ribonuclease. This represents an oxidative stress response mechanism that has not been studied yet.

To investigate the combined effect of phosphatase deficiency and *t*BOOH treatment together, we compared treated and untreated KO strains (KOt vs. KO). We found that the two conditions together resulted in large and important changes in transcription. Genes that were affected by *t*BOOH treatment alone responded to an even greater extent, in addition genes that did not show a significant transcriptional change before, emerged as novel targets.

Expression of 5 genes involved in transport (*HGT1*, *GAP1*, *CDR1*, *TRK1*, and *NAG3)* increased in KOt cells. *HGT1* gene was affected by phosphatase deficiency alone, but in combination with oxidative treatment this effect was amplified. Gene expression of *GAP1*

and *CDR1* increased in WTt cells, but again this effect became more significant in the mutant genetic background. Interestingly, the expression of *TRK1* (K^+ transporter) and *NAG3* (MFS multidrug transporter) genes was significantly enhanced only in the KOt cells. The functional interaction between Trk1 and Ppz1 has been already published, but the fact that this happens at the transcriptional level could only be detected in the cells sensitized by oxidative stress conditions. The function of the *NAG3* multidrug transporter is similar to that of *HGT1* transporter. Thus two pumping mechanisms may support the efflux of the damaging oxidizing agent and survival of KO cells. These results suggest again that CaPpz1 may contribute to drug resistance through the modulation of the transcription of ABC transporters.

Among the genes involved in oxidation-reduction processes, *SOD3*, *SOD4*, *CFL4*, and *AOX2* were sensitively upregulated by *t*BOOH treatment. *SOD3*, *SOD4* and *CFL4* expression was also elevated in WT treated by *t*BOOH, however this response was exaggerated in the Ca*PPZ1* deletion strain. Increased expression of *AOX2* (alternative oxidase) has been published earlier in oxidative stress induced by either H_2O_2 or menadianone. All of these results demonstrate the importance of Aox2 under oxidative stress, suggesting its general protective role.

Downregulation of cell surface proteins under oxidative stress has been previously described. We found that this effect was amplified by deletion of the *CaPPZ1* gene. The mRNA levels of *IFF1* (secreted protein), *PGK1* (phosphoglycerate kinase), and *GPM1* (phosphoglycerate mutase) genes were significantly reduced only in the treated KO cells. The products of the *PGK1*, *GPM1*, and *CDC19* genes mentioned above behave as moonlighting proteins, because they act as well-known metabolic enzymes in the cytosol, but can acquire a novel function at the cell surface where they participate in the symbiont processes. The knowledge of the latter functions may be important from the point of view of developing a new antifungal therapy.

Large expressional changes of genes coding for ribosomal proteins were observed in the stress exposed KO strain. Two distinct patterns were obtained – expression of cytosolic ribosomal protein coding genes went down, while expression of mitochondrial ribosomal protein coding genes went up. Among the genes coding for cytosolic ribosomal proteins, we found 1 ribosomal large subunit gene (*RPL29*), 2 ribosomal small subunit genes (*RPS3*, *RPS8A*), 1 acidic ribosomal protein gene (*RPP1B)*, and the *CAM1* elongation factor gene, for which the absence of phosphatase amplified the previously described suppressive effect

of *t*BOOH treatment. The expression of *EFT2* (translational elongation factor) gene was also significantly reduced. In our proteomic studies, we found that Eft2 may act as a substrate for CaPpz1, because its phosphorylation level was elevated in the KO strain, but we detected no significant change in its gene expression level in the absence of the phosphatase. Thus it is likely that CaPpz1 regulates Eft2 primarily at the posttranslational level, but since oxidative treatment reduced *EFT2* gene expression only in the KO strain, it is possible that an additional transcriptional regulation may also operate under oxidative stress conditions. Obviously the phosphatase deficiency sensitizes the pathogen for the oxidizing agent.

The expression of mitochondrial ribosome associated genes was uniformly elevated in the phosphatase deletion mutant. Increased mRNA levels of *MRPL3* (ribosomal large subunit) and C5_0453W (ribosomal small subunit) were detected in *t*BOOH treated KO cells. The differential regulation of cytosolic vs. mitochondrial ribosome coding genes has been previously noted in a distantly related publication and was explained by the essential function of mitochondrial protein synthesis for the survival of the fungal pathogen under harsh environmental conditions.

Finally, RNA metabolism associated genes were uniformly induced to a lesser or greater extent by *t*BOOH treatment in KO strain. Expression of 4 genes (*BUD22, NSA2, C3_02750W,* and *SPB4*) was already elevated in *t*BOOH treated WT cells, and this rise was enhanced in the absence of the phosphatase. In addition, the expression of *DIM1* rRNA dimethylase and *ENP1* pre-RNA maturation protein increased in KOt cells. As the *BUD22, NSA2, DIM1,* and *ENP1* genes are all involved somehow in the process of pre-rRNA maturation, it is possible that already available pre-ribosomes can assemble at an elevated rate under short-term stress. Our data collectively suggest that in the wild type *C. albicans* CaPpz1 functions as a protective enzyme that helps the fungus to survive osmotic and oxidative stress insults.

Our new findings may contribute to the development of a novel antifungal therapy, because according to our physiological tests, the mild oxidative treatment of the phosphatase deletion mutant cells reduced the cell growth and proliferation. In addition, the deletion (or inhibition) of the phosphatase blocks hypha formation that is normally induced by oxidative stress, and together with the decreased level of virulence factors, diminishes the pathogenicity of the fungus. Meanwhile the fungal cells still remain viable. This is an important issue, as *C. albicans* is part of the normal microbiome. Therefore its complete

removal from the body is not desirable, since it could trigger the proliferation of some potentially more harmful fungal species. We think that the specific inhibition of CaPpz1 with a mild oxidative stress could be a feasible combined treatment of topical fungal infections. Our work presented in my thesis lays down the foundations of further investigations that are necessary to proceed in this direction.

Summary

C. albicans is an opportunistic human pathogen that contains a novel fungus specific Ser/Thr protein phosphatase, called CaPpz1. This enzyme has several important physiological roles such as the regulation of cation homeostasis, cell wall biosynthesis, morphological changes, oxidative stress response, and virulence of the pathogen. In the present study we used modern omic technologies to investigate its further functions and the consequences of phosphatase deletion under oxidative stress.

First, we analyzed the adaptation mechanisms of the phosphatase deletion mutant (KO) compared with WT strain by proteomics. After 2D gel electrophoresis, we identified 25 proteins with mass spectrometry, whose phosphorylation levels and/or protein levels altered in the KO strain. The functional analyses of the affected proteins confirmed the involvement of CaPpz1 in protein synthesis, morphology as well as in oxidative stress response. In addition, we proved a novel role for CaPpz1 in biofilm formation.

Second, we applied transcriptomics to investigate how the *ppz1* KO and WT strains responded to a short oxidative stress induced by *tert*-butyl hydroperoxide (*t*BOOH). We demonstrated that *t*BOOH treatment reduced the growth rate and blocked the proliferation of the fungal cells without affecting their viability or vitality significantly. Furthermore, upon oxidative stress and/or phosphatase deletion the cells responded by elevated antioxidant enzyme activities and oxidized glutathione concentration. Finally we carried out a detailed RNA sequencing study. We found that the lack of CaPpz1 or the oxidative treatment alone, have only a small or moderate effect, but together they generate a robust change in the transcriptome, that indicates a strong positive interaction between the two experimental conditions. Based on gene ontology enrichment analysis we selected 44 genes for further RT-qPCR validations. We confirmed the functions of CaPpz1 in transmembrane transport and oxidation-reduction processes. The expression of genes coding for cell surface proteins and cytosolic ribosomal proteins were downregulated by *t*BOOH, while the amounts of mRNAs associated with transport processes, oxidoreductase activity, and RNA processing were upregulated. All of these changes were enhanced in the KO strain. From these results, we conclude that in *C. albicans* CaPpz1 plays a protective role against oxidative damage. Our data suggest that the specific inhibition of this phosphatase combined with a proper oxidative treatment may be applied as a possible approach to a topical antifungal therapy.

List of publications

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Registry number: DEENK/136/2020.PL
Subject: PhD Publikációs Lista

Candidate: Krisztina Tóthné Szabó
Neptun ID: GXG2A2
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Szabó, K.**, Jakab, Á., Póliska, S., Petrényi, K., Kovács, K., Issa, L. H. B., Emri, T., Pócsi, I., Dombrádi, V.: Deletion of the fungus specific protein phosphatase Z1 exaggerates the oxidative stress response in Candida albicans.
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   PLoS One. 12 (8), 1-21, 2017.
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   IF: 2.766

List of other publications

3. **Szabó, K.**, Kónya, Z., Erdődi, F., Farkas, I., Dombrádi, V.: Dissection of the regulatory role for the N-terminal domain in Candida albicans protein phosphatase Z1.
   PLoS One. 14 (2), 1-27, 2019.
   IF: 2.776 (2018)

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *

Total IF of journals (all publications): 9,043
Total IF of journals (publications related to the dissertation): 6,267

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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