Progress report on the project
„Dissecting the regulatory mechanisms of a fungus specific protein phosphatase"

In accord with our original plan we investigated the physiological role of protein phosphatase Z1 (CaPpz1) in *Candida albicans* during the extended research period (2013-2018) with the support of the National Research, Development and Innovation Office (OTKA/NKFI 108989 grant). We put this enzyme in the focus since according to our previous studies CaPpz1 is fungus specific and has several important physiological functions, thus it can be considered as a potential target for a novel antifungal therapy. As our project progressed we introduced a new research direction: we initiated the studies of the effect of the anti-inflammatory steroid, betamethasone, and the oxidizing agents, menadione and tertiary butyl peroxide (tBOOH) on *C. albicans*. In the last, extension year of the project these two approaches converged as we studied global gene expression changes upon steroid treatment, addition of oxidants, and deletion of CaPpz1 in different combinations. The addition of an unplanned direction and the application of the more advanced RNAseq method instead of the originally designed DNA chip hybridization were beneficial deviations from the original proposal, since they opened up new avenues in research and resulted in interesting, unexpected results. The frequent changes in the list of participants reflected not only the unpredictable changes in the personal career of our co-worker, but were required by the remodeling of our scientific approaches and technologies too. The last/extension year provided sufficient time to complete our original plans and gave us the opportunity to jump into an excitingly new methodology.

The OTKA/NKFI 108989 grant supported us in the publication of 6 research papers in international journals that will be summarized concisely in this report (paragraphs 1-6). In addition we have one manuscript under evaluation (paragraph 7), as well as a host of unpublished data that will be described in paragraph 8.

1. In the first year of the project we adopted a high time-resolution time lapse video microscopy method and introduced a novel digital image analysis to investigate hyphal growth dynamics in different *Candida albicans* strains under near-physiological conditions. We tested the effects of the quorum sensing molecules, and the deletion of specific genes (including *CaPPZ1* that codes for CaPpz1) by this method and found that the processes of the attachment of yeast cells, the yeast-to-hyphal transition and hyphal growth rate are closely related. Our method offers a convenient and reliable way of testing chemicals, including potential drug candidates, and genetic manipulations on the dynamic morphological changes in *C. albicans* (Nagy G, Hennig GW, Petrényi K, Kovács L, Pócsi I, Dombrádi V, Bánfalvi G: Time-lapse video microscopy and image analysis of adherence and growth patterns of *Candida albicans* strains, Appl Microbiol Biotechnol 98 5185–5194, 2014).

2. As a prelude to our new research direction we investigated the effect of the fluorinated glucocorticoid betamethasone on the physiology of *C. albicans.* We reported that betamethasone increased synergistically the anti-candida activity of the oxidative stress generating agent menadione, and suggested that this interaction may be exploited in combination therapies to prevent or cure *C. albicans* infections in the field of dermatology (Jakab Á, Emri T, Sipos L, Kiss Á, Kovács R, Dombrádi V, Kemény-Beke Á, Balla J, Majoros L, Pócsi I: Betamethasone augments the antifungal effect of menadione-towards a novel anti-Candida albicans combination therapy, J Basic Microbiology 54: 1-9, 2015). In the light of our earlier results on the involvement of CaPpz1 in oxidative stress response we

hypothesized that the phosphatase was involved in the elevated oxidation sensitivity of the fungal cells. We tested this possibility by measuring the viability of the candida cells and found that the *cappz1* deletion mutant was exceptionally sensitive to menadione in the presence of the steroid.

3. Based on the above initial findings we extended the investigation of the steroid effect. We initiated an international collaboration and investigated the effect of steroid treatment on the interaction of *C. albicans* with its physiological targets. By using three different epithelial human cell lines we concluded that high-dose application of betamethasone facilitates the interaction, thus it may predispose patients to various candida infections (Jakab Á., Mogavero S., Förster T.M., Jablonowski N., Dombrádi V., Pócsi I., Hube B.: Effects of the glucocorticoid betamethasone on the interaction of Candida albicans with human epithelial cells, Microbiology. 162(12):2116-2125., 2016). The interplay between the steroid, menadione and the phosphatase was also studied, but has not been published yet, and will be described in paragraph 8 of this report, together with the investigation of the synergism between CaPpz1 and another oxidative agent, tertiary butyl peroxide (tBOOH).

4. Parallel to the above physiological investigations we made a significant progress in the field of structural analysis of CaPpz1. In collaboration with a research group from the Brown University, USA, we solved the 3D structure of the well conserved catalytic domain of the enzyme and investigated the possibilities of the specific regulation of the *C. albicans* phosphatase in comparison with the well characterized protein phosphatase 1 catalytic subunit (PP1c). These studies led to the conclusion that the typical PP1c regulator proteins do not modulate the activity of the structurally related CaPpz1 (Chen E, Choy MS, Petrényi K, Kónya Z, Erdődi F, Dombrádi V, Peti W, Page R.: Molecular insights into the fungus-specific serine/threonine protein phosphatase Z1 in *Candida albicans*, mBio 7(4):e00872-16. doi:10.1128/mBio.00872-16., 2016). Thus our X-ray diffraction and biochemical analysis provided evidence for the specific regulation of the fungal enzyme, and suggested that the pharmacological targeting of CaPpz1 can be solved without risking diverse PP1 mediated sideeffects.

5. Next we solved the question of the physiological regulation of CaPpz1 in collaboration with a group from the UAB, Barcelona, Spain. With biochemical and molecular genetic approaches we proved that two *C. albicans* proteins: CaCab3 and CaHal3 are essential subunits of phosphopantothenoylcysteine decarboxylase (PPCDC) and are involved in CoA. In addition, we demonstrated that both of these proteins can interact with CaPpz1 and can inhibit its phosphatase activity *in vitro*. However, under *in vivo* conditions only CaCab3 can act as an important CaPpz1 regulator. Our results suggest that the essential and moonlighting (phosphatase regulating) functions of PPCDC subunits evolved independently (Petrényi K, Molero C, Kónya Z, Erdődi F, Ariño J, Dombrádi V.: Analysis of Two Putative Candida albicans Phosphopantothenoylcysteine Decarboxylase / Protein Phosphatase Z Regulatory Subunits Reveals an Unexpected Distribution of Functional Roles, PLoS One. 11(8):e0160965. doi: 10.1371/journal.pone.0160965., 2016). The interaction of CaCab3 with CaPpz1 may provide us a clue about the natural way of specific phosphatase inhibition, but CaCab3 seems to be an inappropriate drug target due to the universally essential nature of the PPCDC enzyme.

6. The possible interacting partners and substrates of CaPpz1were searched by proteomics and phosphoproteomics analysis of the control and *cappz1* deletion mutant *C. albicans* strains. Our data revealed that many of the proteins whose amount or

phosphorylation level changed upon the deletion of the phosphatase gene were directly or indirectly related to the biofilm formation. Our prediction was confirmed by experiments, thus we revealed an up till now unidentified function of the fungus specific phosphatase (Márkus B, Szabó K, Pfliegler WP, Petrényi K, Boros E, Pócsi I, Tőzsér J, Csősz É, Dombrádi V: Proteomic analysis of protein phosphatase Z1 from *Candida albicans*, PlosOne 12: (8) 1-21, 2017). Biofilm formation of the pathogenic fungus is of interest since it affects the adhesion of the fungal cells to plastic surfaces, including medical implantations and equipment. We also identified two potential substrates of the phosphatase that link CaPpz1 enzyme with protein synthesis.

7. In the paper of Chen et al. (2017) we put forward a model for the regulation of CaPpz1 by the intrinsically unstructured N-terminal domain of the protein. The model is based on biochemical assays and proposes that the N-terminal domain folds back on the globular catalytic C-terminal domain, and blocks several binding sites, including the blocking access of some protein substrates to catalytic cleft. We tested the validity of this assumption by site directed mutagenesis affecting four predicted protein binding sites in the N-terminal domain (Figure 1).

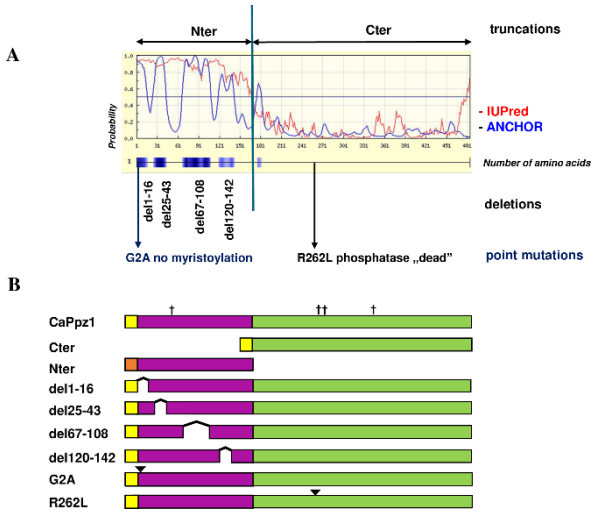

Figure 1. The rationale behind the *in vitro* mutagenesis of the CaPpz1 phosphatase.

A. Bioinformatic analysis of the CaPpz1 protein. The IUPred (red line) and ANCHOR (blue line) software revealed the disordered regions (above 0.5 probability) and four main protein binding sites (blue boxes) in the N-terminal domain of CaPpz1. In the four CaPpz1 deletion mutants, these potential binding sites were eliminated. In addition, two point mutants (the not myristoylated G2A and the inactive R262L) were generated, and the two main domains of the protein (Nter and Cter) were expressed separately.

B. Schematic representation of the bacterially expressed recombinant proteins. The yellow boxes show the residual part of the GST-tag that remains in the bacterially expressed proteins after Prescission protease cleavage, while the brown box represents the 6xHis tag of Nter (note that these features are not present in the proteins expressed in *S. cerevisiae*). The N-terminal domain is violet and the C-terminal domain is green. Point mutations are labeled with a triangle, and four unintentional S to L exchanges due to the special codon usage of *C. albicans* are indicated by crosses.

Expression vectors were constructed to produce the wild type and the mutated forms of the phosphatase in *E. coli*, in *ppz1* deficient *S. cerevisiae*, and in the *cappz1* deletion mutant of *C. albicans*. Unfortunately, the latter did not support persistent fungal expression, and after several unsuccessful attempts we had to drop the testing of the mutations in *C. albicans*. The

rest of the data we decided to publish, and submitted a manuscript to PlosOne (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). In this manuscript we report that according to *in vitro* phosphatase activity assays of the bacterially expressed recombinant proteins the deletion of the N-terminal 1-16 amino acids and the G2A mutation significantly decreased the specific activity of the enzyme. Complementation of the *ppz1 Saccharomyces cerevisiae* deletion mutant strain with the different CaPpz1 forms demonstrated that the point mutations and the N-terminal 1-16 deletion rendered the phosphatase incompetent in the *in vivo* assays. Thus our results revealed the functional significance of the very N-terminal part of the protein in the regulation of CaPpz1. This manuscript is currently under evaluation.

8. According to our original plans the effect of CaPpz1 deletion and the oxidizing agent tBOOH on global gene expression in *C. albicans* was investigated by DNA chip technology. However we experienced some technical problems in our pilot experiments and decided to switch to the more advanced RNAseq method. The advantage of the latter is that the data can be reevaluated whenever the Candida Genome Database is upgraded (corrected and extended), and theoretically allele specific expression patterns can also be determined. After taking into consideration the results of our physiological studies (paragraphs 2 and 3) we extended the scope of the planned experiments by the investigation of the effects of the steroid betamethasone, and the oxidant menadione, in different combinations. These changes of the plans resulted in the triplication of the sample number, but offered a more comprehensive study. Due to some administrative and technical problems we were not able to start the RNAseq studies earlier than the last extension year of the project. We prepared altogether 36 biological samples (12 conditions in triplicates), and obtained a pile of sequencing results within a short period of time. The bottleneck in the project proved to be the analysis of the data. We decided that the effects of the phosphatase KO and the addition of tBOOH will be evaluated first, and the rest of data shall follow suit in the pipeline later.

The quality of the sequencing data was rigorously tested by statistical methods and the correlation between the triplicated biological samples was proven by clustering and PCA analysis. The number of genes exhibiting large (more than twofold) and significant ($p<0.05$) changes are summarized in Figure 2.

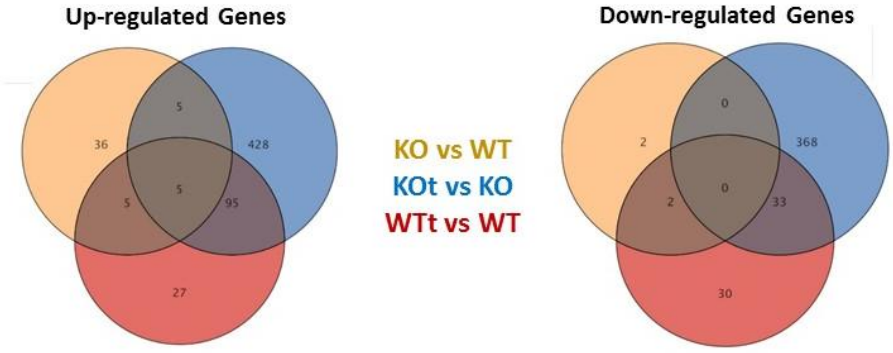

Figure 2. Summary of up-and down-regulated genes in *C. albicans* upon CaPpz1 deletion (KO), tBOOH treatment of the mutant (KOt) and control (WTt), relative to the untreated control (WT) or to the KO strains.

It can be seen from the figure that the phosphatase deletion had only a small effect, tBOOH modulated the gene expression moderately, while the combination of the genetic mutation with oxidative treatment resulted in a robust elevation in the number of genes that were up- or down-regulated. The results indicate a strong synergism and suggest that the function of the phosphatase is to reduce the consequences of oxidative stress.

The affected genes were analyzed according to the function, process, and component related GO terms. We found significant GO term enrichment of the genes involved in: membrane transport, cell surface (including symbiont process), metabolism (including oxidation-reduction process), and translation (including structural component of ribosome and RNA metabolism). Heat maps were generated to identify groups of genes with similar expression pattern changes, and 54 genes were selected for further analysis. The selected genes are associated with transport processes, cell surface, oxidation-reduction, ribosome function and RNA metabolism. In addition 5 genes involved in signal transduction (but not enriched in GO terms) were added to the list. The expression of these genes was investigated by quantitative RT-PCR, and most of the RNAseq data were confirmed. In accord with the overall expression dataset, the KO alone affected just a few genes of transport and oxidoreductase functions. The tBOOH treatment suppressed the expression of cell surface and cellular ribosomal protein related genes; but enhanced transporter, RNA metabolism, rRNA maturation and signal transduction associated genes. Practically in all of the cases the deletion of *CaPPZ1* gene enhanced the response to oxidative stress significantly. When we compared the RNAseq and DNA chip based expression data we found a reasonable correlation between the results of the independent analytical methods (with the exception of a single dataset). Basically the transcript analysis has been completed and could be published as it is, but we would like to extend the data with physiological assays to show the relevance of the gene expression data. We are planning to measure the transport of fluorescent molecules, to monitor rRNA maturation and stability, as well as the changes in the activities of oxidative enzymes under the same conditions. The execution of these assays will need more time, but hopefully it will provide supporting data for a more valuable publication. A manuscript reporting on the above data is planned to be submitted for publication in a half a year time.

As far as the steroid-menadione-phosphatase KO combinations are concerned all of the sequencing data were collected and by their initial analysis we see very similar synergistic interaction between the treatments at the global gene expression level. From the preliminary data the positive interplay between the steroid and oxidative treatment can be forecasted, and the protective effect of the phosphatase from the effects of oxidative stress can be confirmed. However, the data analysis has not been finished yet, and additional experiments are required to support these RNAseq results. Definitely, we have a useful dataset in our hands that shall be published later, in a year or so.

Finally, we endeavored to analyze of the allele specific gene expression. We had to realize that the commonly used data processing pipelines are not suitable for the separation of allele specific sequencing hits of the *C. albicans* transcriptome. We initiated collaboration with the group of Dr Endre Barta, and got the preliminary result telling that the differential expression of a large number of alleles can be predicted from our RNAseq data, however, the statistical analysis of the hits has not been solved yet. Foreseeably, much more time will be needed to sort out this bioinformatic problem. The task was allocated to a new PhD student, but the results cannot be predicted at the moment. If we can get a list of the genes which exhibit significant expression differences between their alleles we can tackle the problem with additional experiments.

Besides the published and to be published scientific papers the project resulted in the completion of several degree and diploma works. Two of or coworkers obtained the PhD degree, three MSc students submitted and defended their dissertations, and ten students received their BSc degrees with the support of this grant. Two of our students were selected from the University of Debrecen for the presentation of their results at the National Scientific Student Conference, where one of them (Krisztina Szabó) earned the first prize in molecular biology, while the other (Fanni Borus) received the third prize in biotechnology.