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Review

The phosphatome of opportunistic pathogen *Candida* species

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ABSTRACT

Several *Candida* species, the best known example of which is *Candida albicans*, are opportunistic human pathogens that are responsible for frequent nosocomial infections. A worrisome aspect of the currently available treatments of candidemia is the steady development of resistance to antifungals among these potentially life threatening fungi. Under these circumstances the search for novel drug targets is a well justified research direction. We propose that the principles of signal transduction therapy by targeting protein phosphatases can be adopted as these enzymes carry out important physiological functions in *Candida*. We demonstrate that *C. tropicalis*, *C. albicans*, *C. dubliniensis*, and *S. cerevisiae* exhibit the largest repertoire of protein phosphatases among the investigated fungi. Together with other opportunistic pathogen *Candida* species and the members of the *Saccharomycetales* order, they expanded their phosphatome by repeated gene duplications. We noted that evolution generated a set of fungus specific phosphatases which can be targeted without the danger of causing undesirable side effects in the human body. Based on the conflicting criteria of effectiveness and selectivity, we identified and characterized 7 phosphatases that are potent virulence determinants and may be utilized as potential antifungal drug targets.

1. Introduction

There are about 200 known *Candida* species that belong to the *Saccharomycetales* order of fungi. Out of them 20 can cause human diseases; the most prominent representative being *Candida albicans* (Poulain, 2015). The peculiarity of this and similar yeasts is that they are common commensals in the human microbiome, about 65 % of the population can be carriers with mild or negligible symptoms (Odds, 1987). However, when the immune system of the affected individuals is weakened or compromised, the opportunistic pathogen turns into a dangerous, sometimes deadly enemy (Perlroth et al., 2007). C.

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albicans was considered to be the fourth most common pathogen responsible for nosocomial infections in USA (Morgan, 2005). According to the latest annual report of Centers for Disease Control and Prevention (https://www.cdc.gov/fungal/diseases/candidiasis/index.html, 2019), the prevalence of systemic candidiasis causing species was the following: *C. albicans* (37 %), *C. glabrata* (27 %), *C. parapsilosis* (14 %), *C. tropicalis* (8 %), and *C. krusei* (2 %). An alarming new development is the emergence of multi-resistant *C. auris* in hospital wards (Forsberg et al., 2019). Between 2013 and 2017, candidemia was diagnosed in about 25,000 patients every year in USA (Tsay et al., 2018). The main target groups were children, especially infants with low weight (Cleveland et al., 2012; Benedict et al., 2018), and elderly people (Barchiesi et al., 2017). It should be added that the mortality rate associated with candidiasis is high, 20–60 % depending on the species (Hirano et al., 2015; Xiao et al., 2019). Consequently, *Candida* infections pose a serious health hazard and a financial drain on the health care systems.

The currently available anti-*Candida* drugs include azole derivatives like fluconazole and echinocandins like caspofungin that inhibit ergosterol (Kathiravan et al., 2012) and cell wall biosynthesis (Lockhart et al., 2012), respectively. Unfortunately, the resistance against these traditional remedies is steadily increasing (Perlin, 2014; Morschhäuser, 2016; Pappas et al., 2016; Robbins et al., 2017). The usefulness of the ergosterol binding polyenes like amphotericin B as a last resort is undermined by toxic side effects (Baginski and Czub, 2009). Under these circumstances the demand for novel potential drug targets and innovative treatments is well justified.

In the quest for new solutions we propose to explore the power of signal transduction therapy that has been successfully used in cancer therapy (Hunter, 2009). The underlying principle is that protein phosphorylation and dephosphorylation together are the most common signaling mechanisms in eukaryotes (Shi, 2009). Protein kinases, the catalysts of the phosphorylation reactions, are very similar to each other as most of them evolved from a single ancestor (Manning et al., 2002). In contrast, protein phosphatases that remove the phosphate group from the proteins are much more divergent. They evolved through convergent evolution, which employed ten distinct protein folds to catalyze seven distinct hydrolytic reaction mechanisms (Chen et al., 2017). The greater structural diversity and the occurrence of fungus specific protein phosphatases (Ariño et al., 2019) offers a possibility to find important and specific targets for rational drug discovery. In fact, the first signal transduction based drugs were the immune suppressors cyclosporin A and FKB506 which, in cooperation with cyclophilin and FKB506B proteins, inhibit the activity of the protein phosphatase calcineurin (Griffith et al., 1995). The human protein phosphatases PPM1D/WIP1 (Ogasawara et al., 2015) and other PPM enzymes (Kamada et al., 2020) as well as PTPN1/PTP1B (Zhang and Zhang, 2007) were also considered as potential drug targets. In the present paper we characterize the *Candida* phosphatome and pinpoint new research directions that could contribute to the control of *Candida* infections.

2. Building up the *Candida* phosphatome

For the compilation of *C. albicans* protein phosphatase sequences, we followed the strategy of Chen et al. (2017) and used the search tools of the CGD database (Skrzypek et al., 2017) with the *S. cerevisiae* dataset of the http://phosphatome.net/3.0/homepage. The latter is an unconventional collection in which the traditional classification schemes have been revised and the list of sequences has been extended by adding new members to the traditional families and by including additional families. We found this novel protein folding based division of the enzymes innovative, and argue that the inclusion of the recently adopted family members extends the base of our search in a useful way. Besides the published 75 members of the *S. cerevisiae* phosphatome (Chen et al., 2017) we also included the gene product of *YNL010W/PYP1* as a potential protein phosphatase (Kuznetsova et al., 2015) as well as that of *SIA1*, a paralog of *DCR2*, identified in our search. Detailed descriptions of the applied bioinformatics methods are given in Appendix A, Text A.1.

The phosphatome of C. albicans

With the aid of 77 *S. cerevisiae* phosphatase sequences, we identified 78 *C. albicans* protein phosphatase genes and proteins (Tab. 1). The nearly equal numbers do not mean that there would be a one-to-one correspondence between the two phosphatomes, rather it is a coincidence due to the independent gene duplications and deletions that compensate each other in the two genomes (Text A.1).

The *C. albicans* phosphatases fall into nine main folds (Fig. 1) since no genuine phosphohistidine specific (PHP-fold) phosphatases can be found in fungi (Chen et al., 2017). All of these families and each family member are described and characterized in Appendix A, Text A.2. Here we give only a concise summary of the classification.

Tyrosine specific and dual specificity protein phosphatases
Unquestionably, the largest and most complex group of phosphatases belongs to the CC1-fold (reviewed by Offley and Schmidt, 2019) that contains 3 typical tyrosine phosphatases (Ptp1-3), 5 plant and fungus specific atypical OCA enzymes (Siw14 is the alias of Oca3, and Oca5 is not a phosphatase) and 4 lipid phosphatases of the SAC1 subfamily. The rest can be classified as dual specificity phosphatases including the yeast myotubularin Ymr1 which has an extended catalytic domain. Within this heterogeneous group we identified 3 new putative phosphatases: (i) Ldh1 is known as a lipid droplet hydrolase in *S. cerevisiae* that has nothing to do with protein dephosphorylation. However, we realized that its *C. albicans* ortholog, the C1_04640W (also termed Ldh1) protein contains not only the common hydrolase domain but an active DSPc-type phosphatase domain, too. (ii) In addition, there are two *C. albicans* genes, C1_09910C (termed *DSP1a*) and its paralog C1_09610W (termed *DSP1b*) that have no *S. cerevisiae* orthologs and code for “low molecular weight” dual specificity phosphatases. The determination of the activities and physiological roles of the novel *Candida* phosphatase domain in Ldh1 and the two DSP isoenzymes requires further experimentation.

Table 1 – Phosphatome of representative fungal species.

Species	*Rozella allomycis*	*Batrachochytrium dendrobatidis*	*Syncephalis fuscata*	*Rhizopus microsporus*	*Schizosaccharomyces pombe*	*Saccharomyces cerevisiae*	*Candida albicans*	*Neurospora crassa*	*Aspergillus nidulans*	*Ustilago maydis*	Core fungal phosphatome
Phylum	*Cryptomycota*	*Chytridiomycota*	*Zoopagomycota*	*Mucoromycota*	*Ascomycota*	*Ascomycota*	*Ascomycota*	*Ascomycota*	*Ascomycota*	*Basidiomycota*	
Fold/Statistics											
CC1	14	14	19	22	16	20	22	14	14	17	9
CC2	2	2	2	2	2	2	2	2	2	2	2
CC3	2	2	2	2	2	3	2	3	3	2	2
HAD	6	8	9	9	7	8	10	9	9	7	5
HP	3	4	4	6	10	18	19	9	13	10	2
PPM	5	5	5	6	7	8	8	7	8	6	4
PPPL	8	9	9	10	11	15	12	10	11	9	8
AP	2	3	1	3	1	1	2	2	2	2	1
RTR	-	-	-	-	1	2	1	1	1	-	-
Phosphatase #	**42**	**47**	**51**	**60**	**57**	**77**	**78**	**57**	**63**	**55**	**33**
Total ORF	6350	8700	8846	11355	5145	6604	6198	10785	10687	6765	-
Phosphatase %	0.66	0.54	0.57	0.53	1.11	1.17	1.26	0.53	0.59	0.81	-

For more detailed information on individual phosphatases, see Tab. B.2. Note that the core phosphatome was determined in the knowledge of the distribution of phosphatases within each fold. The methods used for the construction of this table are described in Appendix A, Text A.1.

As a result of convergent evolution, the enzymes in the CC2- and CC3-fold utilize the same active Cys-based catalytic mechanism as their CC1 counterparts, but have distinctive three-dimensional architectures (Chen et al., 2017).

Serine/threonine specific protein phosphatases

The HAD-fold enzymes use an Asp residue in the hydrolysis of phosphate esters (reviewed by Gohla, 2019). They can be divided into two subfamilies: the typical haloacid dehydrogenase (HAD) domain containing members, some of which are putative protein phosphatases, and the enzymes with a characteristic CPDc domain that can dephosphorylate the C-terminal domain of RNA polymerase II (with the exception of Tim50 that is inactive).

The PPM and PPPL families together are responsible for most of the Ser and Thr specific phosphatase activities in eukaryotic cells (Ingebritsen and Cohen, 1983). They provide another convincing evidence for the convergent evolution, since they use the same binuclear metal ion catalyzed reaction mechanisms, but have different structural folds (Das et al., 1996). All of the metal ion dependent PPM enzymes contain the PP2C domain (reviewed by Ariño et al., 2011). In comparison to *S. cerevisiae*, *C. albicans* lost *PTC3* and gained *PTC8*, consequently both organisms contain 7 PPM phosphatases. The list was completed with the guanylate cyclase Cyr1 which has an inactive PP2C domain. The traditional phosphoprotein phosphatase (PPP) family consists of the most conserved proteins together with their more divergent fungus specific paralogs (reviewed by Ariño et al., 2019). Classical members were identified by traditional biochemical methods, while novel enzymes were revealed by molecular cloning (Cohen, 1997). In line with the suggestion of Chen *et al.* (2017) we extended this group by the addition of Ppn2 as well as two purple acid phosphatases, Dcr2 and Sia1, since all of them encompass the common metallophosphatase domain. With these additions the PPP-like PPPL-fold was introduced which contains 9 classical and 3 unconventional members in *C. albicans* (Fig. 1). The latter modification was justified by two publications (Guo and Polymenis, 2006; Pathak et al., 2007) demonstrating that the *S. cerevisiae* Dcr2 acts as a *bona fide* protein phosphatase in two independent experimental systems.

The recently discovered protein phosphatase Rtr1 (Irani et al., 2016) acts on the C-terminal domain of RNA polymerase II. It stands alone in *C. albicans* with its unique RTR1-fold and still enigmatic reaction mechanism.

Other phosphatases

The active center of the HP-fold phosphatases contains a reactive His residue. In this large family only Yor283w (Kuznetsova et al., 2010) and Tfc7 (Taylor et al., 2013) can dephosphorylate phosphopeptides. The others are mutases and phosphatases affecting sugar or lipid targets, still all of them were taken into consideration for the sake of complete coverage.

The AP-fold containing alkaline phosphatases employ an active Ser in their catalytic cycle. They were also considered because according to a single publication (Donella-Deana et al., 1993), recombinant *S. cerevisiae* Pho8 can dephosphorylate histones and a phosphotyrosine containing peptide *in vitro*. The *PHO8* gene was duplicated in *C. albicans* in such

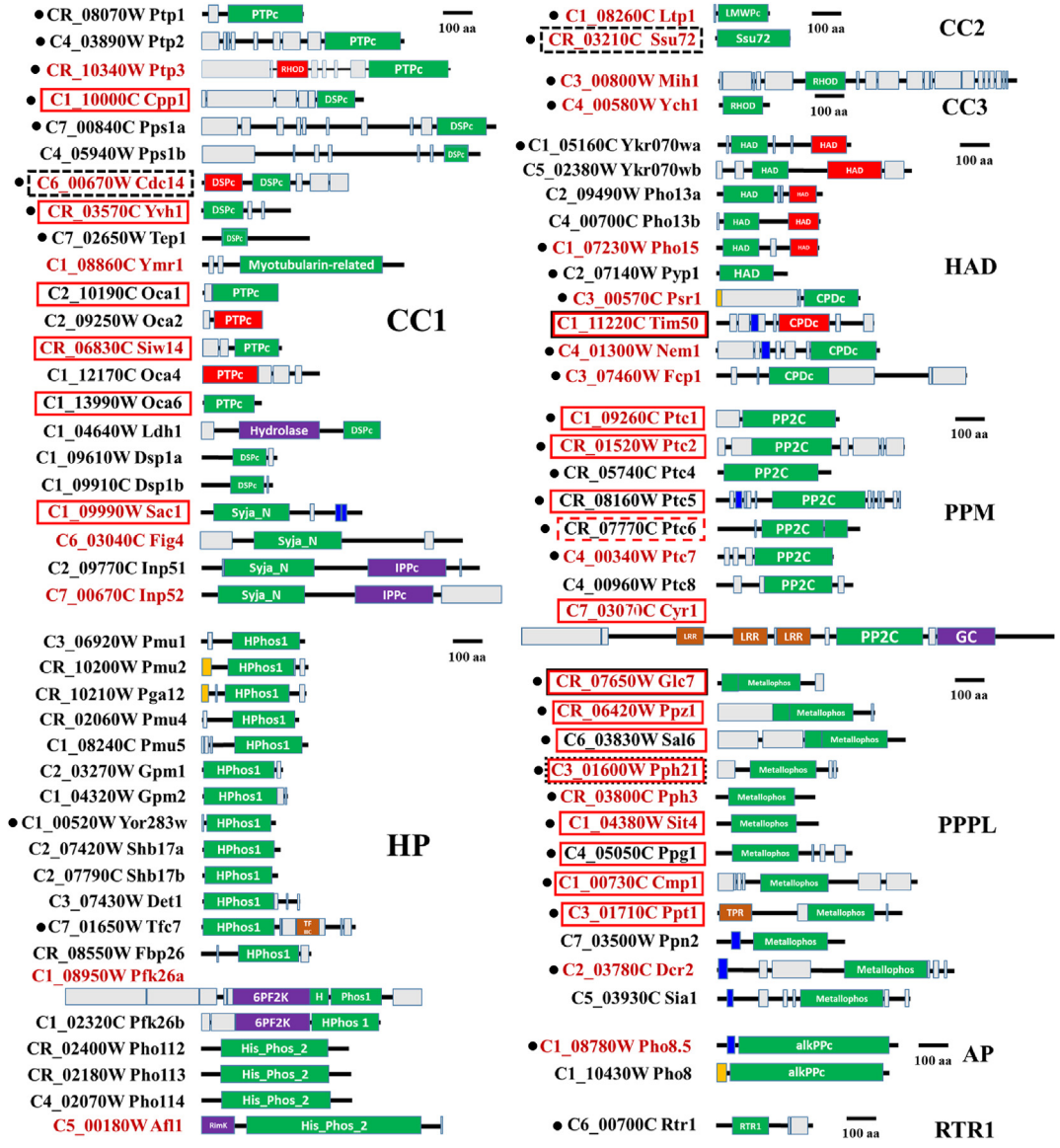

Fig. 1 – Phosphatome of *Candida albicans*. *C. albicans* phosphatases were arranged according to the folding of their catalytic domains. They are identified by their systematic names and standard protein names as described in supplementary Text A.1. Essential gene products are in black boxes and the ones associated with virulence are in red boxes. Full line borders of boxes indicate strong evidence, broken lines denote contradictory data, and dotted line shows circumstantial evidence. The members of the fungal core phosphatome are highlighted with brick red names. *Bona fide* protein phosphatases are labeled with a dot in front of the names (for details of identifications, see Tab. B.2). The schematic structures of the corresponding proteins are depicted to the right. The polypeptide backbones are shown as black lines. Green and red bars on the lines represent active and inactive phosphatase catalytic domains, respectively. Additional active non-phosphatase domains are in violet, and non-catalytic domains are in brown. Transmembrane regions are blue, signal peptides are ochre, and all of the unstructured, coiled or disordered segments are presented as grey bars. Scales denote the size of a peptide of 100 amino acid residues. Protein domains were labeled as: PTPc - Protein Tyrosine Phosphatase, RHOD - rhodanase domain, DSPc - Dual Specificity Phosphatase, Syja_N – N-terminal synaptojanin, IPPc - Inositol Polyphosphate Phosphatase, LMWPc - Low Molecular Weight protein tyrosine Phosphatase, SSU72 - Suppressor of sua7-1 gene 2, HAD - HaloAcid Dehydrogenase, CPDc - catalytic domain of CTD-like phosphatases, HPhos1- Histidine phosphatase superfamily (branch 1), TFIIIC - TFIIIC subunit triple barrel domain, 6PF2K - 6-phosphofructo-2-kinase, His_Phos_2 - Histidine phosphatase superfamily (branch 2), RimK – Rim Kinase-like ATP-grasp domain, PP2C - Protein phosphatase 2C, LRR - Leucine-Rich Repeats, GC – Guanylate Cyclase, Metallophos - Calcineurin-like phosphoesterase, TPR - TetratricoPeptide Repeats, alkPPc – alkaline PhosPhatase, RTR1 - Rtr1/RPAP2 family.

Table 2 – Phosphatome of *Candida* species.

Species	*C. albicans*	*C. albicans*	*C. dubliniensis*	*C. tropicalis*	*C. orthopsilosis*	*C. parapsilosis*	*L. elongisporus*	*D. hansenii*	*C. guilliermondii*	*C. auris*	*C. lusitaniae*	*C. lusitaniae*	*C. glabrata*
Strain	SC5314	WO-1	CD36	MYA-3404	Co 90-125	CDC317	NRLL YB-4239	CBS767	ATCC 6260	B8441	ATCC 42720	CBS 6936	CBS138
Fold/Statistics													
CC1	22	21[a]	22	21	19	19	19	21	21	19	20	20	19
CC2	2	2	2	2	2	2	2	2	2	2	2	2	2
CC3	2	2	2	2	2	2	2	2	2	2	2	2	2
HAD	10	10	10	10	10	10	9	9	9	9	9	9	8
HP	19	19	19	20	16	16	17	16	15	13	13[b]	14	16
PPM	8	8	8	8	8	8	8	8	8	7[c]	8	8	8
PPPL	12	12	12	12	13	14[d]	12	12	12	12	11	11	12
AP	2	2	2	3	2	2	2	2	1	1	1	1	1
RTR	1	1	1	1	1	1	1	1	1	1	1	1	2
Phosphatase #	**78**	**77**	**78**	**79**	**73**	**74**	**72**	**73**	**71**	**66**	**67**	**68**	**70**
Total ORF	6198	5752	5862	6254	5678	5837	5799	6272	5920	5417	5936	5537	5294
Phosphatase %	1.26	1.34	1.33	1.26	1.29	1.27	1.24	1.16	1.20	1.22	1.13	1.23	1.32

More detailed information on phosphatases in each species is provided in Supplementary, Tab. B.1.
a The Pps1a ortholog of *C. albicans* WO-1 (formerly CAWG_05441) has been recently deleted from the Ensembl database.
b The ortholog of Shb17 is missing from the *C. lusitaniae* ATCC 42720 but it is present in strain CBS 6936.
c Pct6 is missing from the databases of *C. auris* B8441 but it is present in the very similar *C. auris* B11221 strain.
d Note that the PPN2 gene was duplicated in *C. parapsilosis* that makes it different from the closely related *C. orthopsilosis*.

a way that the *Candida* Pho8.5 paralog is more similar to the budding yeast Pho8.

The Candida *phosphatome*

After establishing the *C. albicans* phosphatome we used this dataset to reveal the orthologous proteins in 10 additional pathogenic *Candida* species (Tab. 2), and obtained altogether 947 protein sequences that were used to construct the family trees for each fold (Fig. 2). It should be noted that the Candida Genome Database (CGD, Skrzypek et al., 2017) contains a "Homologs" section for every *C. albicans* gene that summarizes the most closely related orthologs in different *Candida* and fungal species. In general terms, our data are in good correlation with this database with the exception of two differences: (i) CGD does not cover the CBS6936 strains of *C. lusitaniae,* (ii) and more importantly, does not record paralogs, thus overlooking gene duplications that are quite common among phosphatases (Fig. 2). During the BLAST searches we realized that the quality of the different genome projects was not uniform; in some of the databases we found a few uncertain or incomplete sequences (Tab. B.1). To test the effect of possible sequencing errors, we compared the SC5314 and WO-1 strains of *C. albicans* as well as the ATCC 42720 and CBS 6936 strains of *C. lusitaniae* (Tab. 2). We found a difference of ± 1 phosphatase/species that can be attributed to sequencing problems. Other differences are related to gene deletions/duplications which happen most frequently in the HP-fold. Despite individual differences, the abundance of phosphatase genes is quite stable. In the pathogenic *Candida* species, they account for more than 1 % of all ORFs. Obviously, *C. glabrata* is an outsider since contrary to the others, it underwent whole genome duplication and does not follow the distinctive CTG codon usage (Butler et al., 2009). According to its genetics it is more similar to the nonpathogenic *S. cerevisiae* that has a somewhat larger phosphatome (Tab. 1 and 2). Note that throughout this review we consistently applied the rules of the *S. cerevisiae* nomenclature for all genes and proteins irrespective of their origins.

3. Selection of targets for drug discovery

For the identification of suitable drug targets in the *Candida* phosphatome, we relied on the traditional "magic bullet" paradigm of antimicrobial chemotherapy (Williams, 2009), anticipating that a drug should hit a physiologically significant and specific target in the pathogen without harming the human body.

Selection of physiologically significant phosphatases

To find the most significant protein phosphatases of *Candida*, we were looking for essential genes in the CGD, and got a somewhat disappointing result (Fig. 1). Out of the 78 candidates, only the *GLC7* (Hu et al., 2012) and *TIM50* (Becker et al., 2010) genes proved to be absolutely vital. Conflicting results were obtained for *CDC14* (Clemente-

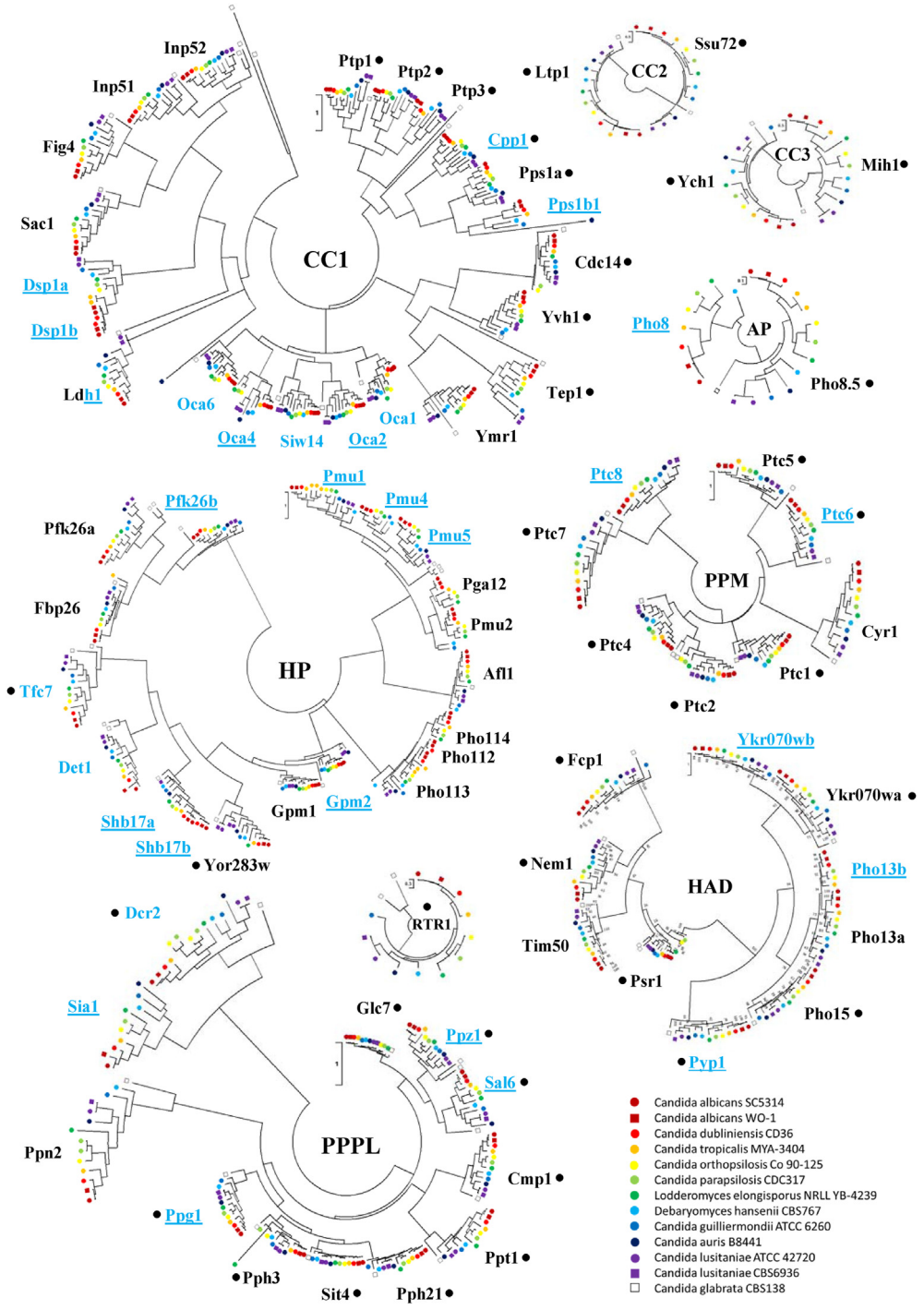

Fig. 2 – Phylogenetic analysis of *Candida* protein phosphatases. The phylogenetic relationships between the amino acid sequences of protein phosphatases taken from 13 strains of 11 *Candida* species are presented in 9 family trees representing the main phosphatase folds. Each color-coded symbol (see insert) in a given branch corresponds to an ortholog of the *C. albicans* phosphatase whose standard protein name labels the branch. Underlined blue protein names highlight the fungus specific enzymes, while blue names mark the phosphatases that are not fungus specific, but have no animal orthologs. The scales indicate evolutionary distances. *Bona fide* protein phosphatases are labeled with a dot as in Fig. 1. The systematic names for all of the proteins as well as the branch statistics for each node can be found in supplementary Tab. B.1. and Fig. C.1.

Blanco et al., 2006, vs. Segal et al., 2018) and *SSU72* (Segal et al., 2018, vs. Xu et al., 2007), while only circumstantial evidence supports the essential nature of *PPH21* (personal communication by Mitchell, 2009). The contradictions expose a technical problem in establishing the viability of a null mutant, namely, the results are substantially affected by the genetic background and by the testing conditions. Next, we searched which phosphatases were involved in the virulence of the

Table 3 – *Candida* protein phosphatases selected as potential antifungal drug targets.

Standard Name	Protein Fold	Phosphatase Classification	Best Human Ortholog			
			Protein ID[b]	Name[b]	E-value	Note
[a]Cpp1	CC1	DSP	Q6P9C2	Dusp9	1.5e-9	Dual specificity protein phosphatase
			F8VW29	Dusp6	1.8e-9	Tyrosine protein phosphatase
Oca1	CC1	Atypical DSP	Q9UL68	Myt1l	3.5e-2[c]	Myelin transcription factor 1-like protein
Siw14	CC1	Atypical DSP	O14522	Ptprt	2.2e-2[c]	Receptor-type tyrosine protein phosphatase
Oca6	CC1	Atypical DSP	Q6UXP6	LKHP9428	1.7e0[c]	Unknown protein
[a]Ppz1	PPPL	Novel PPP	P62136	Pp1a	5.4e-141	Serine/threonine protein phosphatase PP1-alpha
[a]Sal6	PPPL	Novel PPP	P36873	Pp1g	3.4e-131	Serine/threonine protein phosphatase PP1-gamma
[a]Ppg1	PPPL	Novel PPP	P60510	Pp4c	1.1e-107	Serine/threonine protein phosphatase 4

a Indicates *bona fide* protein phosphatases.
b Protein IDs and Names are from UniProt (https://www.uniprot.org).
c E-values larger than e−5 denote insignificant sequence similarity. For more details on target analysis, see Text A.1.

pathogen. By screening the CGD "Phenotype" sections we found relevant references for the decreased virulence of deletion mutants suggesting the direct or indirect involvement of 20 phosphatases in the virulence of *C. albicans* (Fig. 1). Note that three of the genes that are required for full virulence (*TIM50, GLC7,* and *PPH21*) were found essential in other experiments, and 14 of them code for *bona fide* protein phosphatases. The results on the *PTC6* gene are somewhat uncertain as its deletion had no detectable consequences (Yu et al., 2010). Furthermore, the epistatic genetic relationship between *PTC6* and *PTC5* has been demonstrated in several experiments (Zhao et al., 2012), still *PTC5* was not able to compensate the absence of *PTC6* in an insect cell infection test of virulence (Hanaoka et al., 2008). The systematic overexpression of protein phosphatase catalytic subunits in *C. albicans* revealed that 11 gene products suppressed filamentation (Bar-Yosef et al., 2018). We noted that 7 of these morphogenic phosphatases (*PTP3, PTC1, PTC5, GLC7, PPG1, SAL6/PPQ1*, and *PPZ1*) are required for full virulence (Fig. 1). Of course, we have to keep in mind that different genetic backgrounds and experimental approaches can affect the results of virulence tests, too.

Because of the experimental uncertainties, we also scrutinized the significance of phosphatases by investigating their conservation in fungi from the ancient *Rozella allomycis* to the more recent *Ustilago maydis*. For this purpose, we selected 10 sequenced fungal species of 9 orders from the MycoCosm database (Grigoriev et al., 2014). By searching of the species-specific sequence databases, we determined the core fungal phosphatome that consists of 33 phosphatases (Tab. 1 and Tab. B.2). We hypothesized that the core phosphatases must have important functions and indeed all, of the 5 essential and most (14 out of 20) of the virulence determinant genes coincided with the core phosphatome (Fig. 1). Thus, our evolution-based search confirmed the results of genetic screens and reassured that 16 *Candida* phosphatases are significant, 13 of which can dephosphorylate protein substrates.

The comparison of fungal phosphatomes revealed that the number of phosphatases is not directly proportional to the complexity of an organism. The minimal set of around 42 in *R. allomycis* is somewhat larger than the core phosphatome. This number gradually increases to about 60, and then stabilizes independently of the genome size. It means that approximately 60 phosphatases are not only sufficient but also optimal for controlling the important dephosphorylation reactions in most of the fungi. However, the representatives of *Saccharomycotina* exceed this limit and keep expanding their phosphatomes by repeated gene duplications, first of all in the HP-, CC1-, and PPPL-folds. The largest number of protein phosphatases (79) was found in *C. tropicalis*. Concerning *S. cerevisiae* and *C. glabrata*, this tendency can be partially explained by whole genome duplication, but certainly, individual gene deletions and duplications have to be taken into consideration, too. It is conceivable that in the pathogenic *Candida* species (Tab. 2), the extra phosphatases can contribute to the fine-tuning of signaling pathways and may be advantageous for the adaptation to the environmental effects associated with the opportunistic pathogenic lifestyle (Brown et al., 2014). Furthermore, the expansion of the phosphatomes and the consequently enhanced genetic variability may aid the evolution of specific pathogenic mechanisms characteristic to *Candida* (Mayer et al., 2013).

Selection of fungus specific phosphatases

Next, we investigated the distribution of *Candida* phosphatase orthologs in a wide range of living organisms (Tab. B.3) in order to identify the enzymes which are present only in fungi. The 23 fungus specific phosphatases (and the fungus specific phosphatase domain of Ldh1) are highlighted in Fig. 2 to demonstrate their phylogenetic relationships to their more widely distributed paralogs that were most probably the starting points of their evolution. In a less stringent search we got 6 additional phosphatases that are not fungus specific, but have no animal orthologs (Fig. 2). In these two categories there are no more than 8 *bona fide* protein phosphatases, indicating that the majority of the fungus specific phosphatases are metabolic enzymes.

In accord with the "magic bullet" analogy, the union of the information gathered in Figs 1 and 2 defines potential drug targets. It is clear that the requirements of effectiveness and selectivity work against each other; the most important proteins are well conserved, while the more species specific ones have a shorter evolutionary history to gain important independent functions. As the specific members of the most variable HP-fold are not physiologically significant, the best

candidates stem from the CC1- and PPPL-folds and include Cpp1, the three active enzymes of the OCA subfamily, as well as the novel PPP phosphatases Ppz1, Sal6, and Ppg1 (Tab. 3). Importantly, all of the selected targets are conserved in all of the 11 pathogenic *Candida* species scrutinized (Fig. 2).

4. Characterization of putative drug targets

In the following sections we briefly describe the 7 phosphatases that were selected as putative targets of antifungal therapy, and check if there are any human proteins with similar structural elements that may compromise the suggested phosphatase inhibition based pharmacological intervention (Tab. 3). This is an important test since despite of their fungus specificity (i.e. they are not the best orthologs of human phosphatases) they can still have distantly related human orthologs (Text A.1).

Cpp1

C. albicans Cpp1 is the ortholog of the *S. cerevisiae* MAP kinase phosphatases Msg5 and Sdp1 (Csank et al., 1997) that are known to cooperate with the classical Ptp2 and Ptp3 tyrosine phosphatases in the modulation of extracellular signal regulated pathways (Martín et al., 2005). It is a potent regulator of hyphal growth and a potential virulence factor (Csank et al., 1997) that counteracts the effects of the Cek1 MAP kinase and connects the Cek1 and Hog1 signaling pathways (Deng and Lin, 2018). The reduced virulence of the null mutant was confirmed in two independent infection models (Guhad et al., 1998; Brennan et al., 2002). This dual specificity protein phosphatase belongs to the core phosphatome of fungi. Its catalytic domain exhibits limited sequence homology with the human dual specificity phosphatases Dusp9 and Dusp6, but it has a specific N-terminal segment that may be considered as a suitable drug target (Text A.1).

OCA subfamily

Fungi and plants contain a unique subfamily of OCA (Oxidant-induced Cell-cycle Arrest) phosphatases that were termed atypical DSPs (Romá-Mateo et al., 2011). In *S. cerevisiae* Oca1, Swi14/Oca3, and Oca6 are supposed to be active enzymes. Oca2 is a close relative of Oca1 but has no essential Cys in its catalytic center, while Oca4 appears to be a derivative of Siw14/Oca3 that got inactivated by the deletion of its entire catalytic motif. The overexpression of either the inactive Oca2 or the active Oca6 proteins compromised signaling through the cell wall integrity pathway (Sacristán-Reviriego et al., 2015). All of the five OCA subfamily members are present in all *Candida* species and the deletion of *OCA1*, *SIW14/OCA3*, and *OCA6* genes resulted in defective virulence *of C. albicans* (Hanaoka et al., 2008). They look like excellent species specific targets since they have no orthologs in *H. sapiens* (Tab. 3). This promising possibility should encourage further investigations of their physiological functions in *Candida*.

Ppz1

In budding yeast there are two similar fungal specific Glc7-like protein phosphatases: Ppz1 and Ppz2 (Ariño et al., 2019). *C. albicans* has a single Ppz1 ortholog which belongs to the core fungal phosphatome and is involved in the monovalent cation transport, in the cell wall integrity (Ádám et al., 2012; Szabó *et al.*, 2019a, 2019b), and probably in the transport of phosphate, glycerophosphoinositol, and glucose as well (Szabó et al., 2019a). Its role in the oxidative stress response was reported (Leiter et al., 2012) and confirmed in a transcriptomic study (Szabó et al. 2019a), while its association with protein synthesis and biofilm formation was deduced from proteomics data (Márkus et al., 2017). Two recent publications on the overexpression of Ppz1 in *S. cerevisiae* support the role of this phosphatase in translation (Calafi et al., 2020), oxidative stress and cell cycle regulation (Velázquez et al., 2020). In a homozygous *ppz1* deletion mutant, the attachment of the yeast cells to solid surface was delayed and the rate of yeast-to-hyphae transition was reduced (Nagy et al., 2014). The null mutant exhibited decreased competitive fitness (Noble et al., 2010) and reduced virulence (Ádám et al., 2012). In agreement, the *A. fumigatus* ortholog of Ppz1 is also a virulence factor (Muszkieta et al., 2014). Thus, PPZ phosphatases are promising drug targets. Although the C-terminal catalytic domain of *C. albicans* Ppz1 is similar to the catalytic subunit of human protein phosphatase 1 alpha, important Ppz1-specific structural elements were identified in its tertiary structure that may serve as fungus specific targets of pharmacological intervention (Chen et al., 2016). In addition, the unique N-terminal regulatory domain of the phosphatase (Szabó et al., 2019b) can also be considered as a specific target (Text A.1). The synergistic interaction between phosphatase deletion and oxidative stress treatment (Szabó et al., 2019a) suggests that the combination of a putative Ppz1 inhibitor with an oxidizing agent may be a useful way of controlling *Candida* in topical applications.

Sal6/Ppq1

This novel Glc7-like protein phosphatase was termed either Ppq1 (Protein Phosphatase Q1, by Chen et al., 1993) or Sal6 (Suppressor of Aac2 Lethality 6, by Vincent et al., 1994) in *S. cerevisiae*. Relatively little is known about the yeast enzyme: it downregulates mating factor signaling by the dephosphorylation of unidentified proteins in the Fus3 MAP kinase pathway (Shim and Park, 2014; Sacristán-Reviriego et al., 2015) and may replace Glc7 phosphatase in some protein complexes (Ariño et al., 2019). The *SAL6* gene was found only in the *Saccharomycetes* class (Tab. B.2) suggesting that it evolved from *GLC7* much later than *PPZ1*. The only important information about *C. albicans* Sal6 is the decreased virulence of its null mutant (Hanaoka et al., 2008). The C-terminal catalytic domain of Sal6 exhibits a high sequence similarity to the catalytic subunit of human protein phosphatase 1 gamma isoform; however its specific N-terminal domain (that is different from that of Ppz1) may be exploited in drug discovery.

Ppg1

Ppg1 (Protein Phosphatase involved in Glycogen accumulation 1) received its name since the glycogen content of the *ppg1* deletion mutant was less than normal in *S. cerevisiae* (Posas et al., 1993). It was found essential for the dephophorylation of the mitophagy receptor Atg32 to inhibit mitosis (Furukawa et al., 2018), and the *S. pombe* ortholog Ppa3 is part of the SIN (Septation Initiation Network) complex (Singh et al., 2011). In *C. albicans* the *ppg1* null mutant exhibited compromised filamentous growth (Noble et al., 2010) and reduced virulence (Albataineh et al., 2014). The primary structure of the N-terminal catalytic domain of this enzyme is closely related to that of the catalytic subunit of protein phosphatase 4 in *H. sapiens*, still the unusual C-terminal domain and the strictly restricted fungal distribution of Ppg1 may make it a possible antifungal target.

5. Conclusions

From the comparison of fungal model organisms we concluded that *Saccharomycotina,* including many pathogenic *Candida* species, are exceptional as their phosphatomes keep expanding *via* repeated gene duplications. This still ongoing phenomenon has contributed to the generation of several novel fungus specific phosphatases. Unfortunately, in many cases only limited information is available on the physiological significance of these novel enzymes either because they did not have sufficient time for functional specialization, or because they have not been studied extensively yet. In spite of these difficulties we were able to pinpoint 7 phosphatases that exhibit fungus-specific phylogenetic distribution and are involved in the virulence of *C. albicans*. Three of the OCA family members are highly specific since their genes were lost in the animal kingdom; however, they have not been well characterized. In contrast, the other four candidates have proven protein phosphatase activity and can modulate important signaling pathways and/or physiological processes in the pathogenic *Candida* species. Interestingly, all of the latter evolved *via* the same mechanism: exon shuffling attached an intrinsically disordered regulatory domain to the N- or C-terminal end of their parental catalytic domain (Text A.1) and generated novel fusion proteins with distinctive interactions, regulation and physiological functions which differentiate them from their parents. Based on our systematic analysis we conclude that evolution produced several novel protein phosphatases that are advantageous for the virulence of the opportunistic pathogens, but at the same time may offer promising handles in controlling candidemia.

It should be mentioned that our pipeline designed for the identification of significant and specific protein phosphatase domains can be readily used for searching any other putative drug targets in pathogens and it can be extended by a novel aspect. Namely, the PPPL-fold phosphatases tend to operate as multisubunit holoenzymes built up from a catalytic and one or more regulatory polypeptides (Brautigan, 2013). In this case it is possible to divide the two contrasting requirements between the interacting partners; for example, a conserved and essential catalytic subunit can bind to unique fungus specific regulatory subunits. The usefulness of such an approach has been exemplified by calcineurin, a calcium/calmodulin-dependent protein phosphatase that is involved in the virulence and drug resistance of several pathogenic fungi (Juvvadi et al., 2017).

Role of authors

Krisztina Szabó: Carried out most of the bioinformatics analysis, prepared documentation, organized references, and wrote the Methods section of the supplement.

Márton Miskei: Helped in the construction of family trees.

Ilona Farkas: Took part in BLAST searches, wrote sections of the supplementary texts, and revised the manuscript.

Viktor Dombrádi: Formulated the concept, supervised documentation, designed and wrote the article.

Declaration of competing interest

No issues of conflicting interest have been declared or identified.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbr.2020.12.002.

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