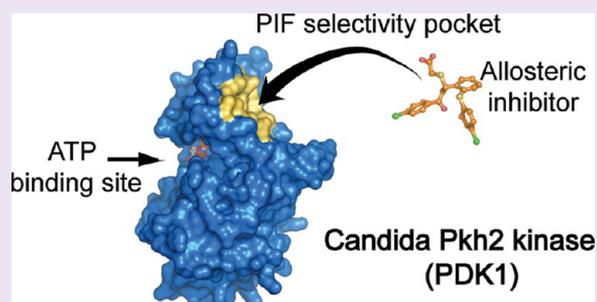


PIF-Pocket as a Target for *C. albicans* Pkh Selective InhibitorsDaniel Pastor-Flores,<sup>†</sup> Jörg O. Schulze,<sup>†</sup> Anna Bahí,<sup>‡,§</sup> Romina Giacometti,<sup>||</sup> Jofre Ferrer-Dalmau,<sup>‡,§</sup> Susana Passeron,<sup>||</sup> Matthias Engel,<sup>⊥</sup> Evelyn Süß,<sup>†</sup> Antonio Casamayor,<sup>\*,‡,§</sup> and Ricardo M. Biondi<sup>\*,†</sup><sup>†</sup>Research Group PhosphoSites, Medizinische Klinik 1, Universitätsklinikum Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany<sup>‡</sup>Departament de Bioquímica i Biologia Molecular, Facultat de Veterinària, Universitat Autònoma de Barcelona, Cerdanyola 08193, Barcelona, Spain<sup>§</sup>Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona, Cerdanyola 08193, Barcelona, Spain<sup>||</sup>Cátedra de Bioquímica, Facultad de Agronomía, Universidad de Buenos Aires, C1417DSE Buenos Aires, Argentina<sup>⊥</sup>Pharmaceutical and Medicinal Chemistry, Saarland University, P.O. Box 151150, D-66041 Saarbrücken, Germany

## Supporting Information

**ABSTRACT:** The phosphoinositide-dependent protein kinase 1, PDK1, is a master kinase that phosphorylates the activation loop of up to 23 AGC kinases. *S. cerevisiae* has three PDK1 orthologues, Pkh1–3, which also phosphorylate AGC kinases (e.g., Ypk, Tpk, Pkc1, and Sch9). Pkh1 and 2 are redundant proteins involved in multiple essential cellular functions, including endocytosis and cell wall integrity. Based on similarities with the budding yeast, the Pkh of fungal infectious species was postulated as a novel target for antifungals. Here, we found that depletion of Pkh eventually induces oxidative stress and DNA double-strand breaks, leading to programmed cell death. This finding supports Pkh as an antifungal target since pharmacological inhibition of Pkh would lead to the death of yeast cells, the ultimate goal of antifungals. It was therefore of interest to further investigate the possibility to develop Pkh inhibitors with selectivity for *Candida* Pkh that would not inhibit the human ortholog. Here, we describe *C. albicans* Pkh2 biochemically, structurally and by using chemical probes in comparison to human PDK1. We found that a regulatory site on the *C. albicans* Pkh2 catalytic domain, the PIF-pocket, diverges from human PDK1. Indeed, we identified and characterized PS77, a new small allosteric inhibitor directed to the PIF-pocket, which has increased selectivity for *C. albicans* Pkh2. Together, our results describe novel features of the biology of Pkh and chemical biology approaches that support the validation of Pkh as a drug target for selective antifungals.



## INTRODUCTION

Fungal organisms are responsible for life-threatening systemic infections, especially in immunocompromised patients. Current antifungals most often target features that are present in the infectious organisms but absent in humans (e.g., components of the cell wall and cell membranes). However, it may be possible to target proteins that have related counterparts in the human host, if sufficient specificity for the fungal target can be achieved or if the inhibition of the human counterpart does not produce major side effects. One such example is Pkh, the fungal ortholog of the phosphoinositide-dependent protein kinase 1 (PDK1), which has been postulated as an antifungal drug target.<sup>1</sup> In multicellular organisms, PDK1 phosphorylates the activation loop of the AGC family of protein kinases<sup>2</sup> (named after three representatives of the group, PKA, PKG, and PKC).<sup>3</sup> PDK1 and a subset of AGC kinases contain a regulatory site in the catalytic domain, termed PIF-pocket.<sup>4</sup> The site is used by PDK1 to interact with some substrates and mediates the regulation of the activity of many AGC kinases.<sup>3</sup> Thus, the PIF-pocket mediates the activation by phosphorylation of diverse

AGC kinases<sup>5,6</sup> and also the allosteric inhibition by N-terminal domains.<sup>7–9</sup> Along the years, we and others have developed small molecules directed to the PIF-pocket that either activate or inhibit different AGC kinases (recently reviewed in ref 3). However, we have not yet identified allosteric inhibitors of PDK1 or allosteric molecules targeting the PIF-pocket of any yeast protein kinase.

The genome of the yeast *S. cerevisiae* contains three genes (termed *PKH1–3*) that encode protein kinases whose catalytic domains are approximately 70% identical to human PDK1 and, similar to PDK1, possess a long C-terminal noncatalytic region. *PKH1* and *PKH2* are functionally redundant genes with essential functions because spores deleted for both genes, but not for either single one, are not viable.<sup>10</sup> Most of the data gained about the effects of the lack of Pkh has been obtained by using *PKH2* deleted cells (*pkh2Δ*) carrying a thermolabile

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Table 1. Yeast Strains Used in the Present Study

strain	relevant genotype	PKH gene expressed with doxycycline	source/ref.
CML476	<i>MATa ura3-52 leuΔ1 his3Δ200 GAL2 CMVp(tetR'-SSN6)::LEU2 trp1::tTA</i>	<i>PKH1, PKH2, PKH3</i>	36
MB002	<i>MATa CML476 KanMX4-(tetO<sub>7</sub>):PKH2</i>	<i>PKH1, PKH3</i>	present study
MB005	<i>MATa CML476 KanMX4-(tetO<sub>7</sub>):PKH2 pkh1::HIS3</i>	<i>PKH3</i>	present study
SDP7	<i>MATa CML476 KanMX4-(tetO<sub>7</sub>):PKH2 pkh3::nat1</i>	<i>PKH1</i>	present study
SDP8	<i>MATa CML476 KanMX4-(tetO<sub>7</sub>):PKH2 pkh1::HIS3 pkh3::nat1</i>	-	present study
SDP10	<i>MATa ura3-52 leuΔ1 his3Δ200 GAL2 CMVp(tetR'-SSN6)::LEU2 erg6::TRP1</i>	<i>PKH1, PKH2, PKH3</i>	present study
YAB100	<i>MATa CML476 KanMX4-(tetO<sub>7</sub>):PKH2 pkh1::HIS3 mca1::nat1</i>	<i>PKH3</i>	present study
BY4741	<i>MATa his3Δ1 leu2Δ met15Δ ura3Δ</i>	<i>PKH1, PKH2, PKH3</i>	37
Y04300	<i>BY4741 pkh3::KanMX4</i>	<i>PKH1, PKH2, PKH3</i>	37
YPP64T	<i>MATa ade2 trp1-901 leu2-3,112 lys2-801am his3Δ200 lys2::(lexAop)4-HIS3 ura3-52::ura3 (lexAop)8-lacZ pdr5::loxP, snq2::loxP, yor1::loxP, pdr1::loxP, pdr3::loxP</i>	<i>PKH1, PKH2, PKH3</i>	present study
YPP66	<i>MATa Gal4-452 Gal80-538 ade2-101 his3-Δ200 leu2-3,112 trp1-901 ura3-52 lys2-801 URA3::UAS<sub>GAL1</sub>-LacZ lys2::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-HIS3 cyh<sup>R</sup> pdr5::loxP, snq2::loxP, yor1::loxP, pdr1::loxP, pdr3::loxP</i>	<i>PKH1, PKH2, PKH3</i>	present study

Pkh1 protein (encoded by the *pkh1*<sup>D398G</sup> allele) and incubation at the restrictive temperature (37 °C).<sup>11</sup> Pkh3 is a third and more distantly related protein that was identified as a multicopy suppressor of the growth defect of *pkh1*<sup>D398G</sup> *pkh2Δ* mutant cells at 37 °C.<sup>11</sup> In contrast to what occurs with the *pkh1Δ* *pkh2Δ* inviable cells, the *pkh1Δ* *pkh3Δ* and the *pkh2Δ* *pkh3Δ* double mutant strains grow normally.<sup>12</sup>

Similarly to mammalian PDK1, the yeast Pkh proteins exert pleiotropic effects by phosphorylating the activation loop of diverse AGC protein kinases substrates. This phosphorylation is indispensable for the activity of at least Ypk1, Ypk2, Pkc1, Sch9, Tpk1, and Tpk3 protein kinases.<sup>12,13</sup> Phosphorylation of Pkc1 by Pkh is crucial for maintaining cell wall integrity (CWI) by the activation of the SlT2MAPK signaling cascade<sup>11</sup> and for the regulation of the processing body (P-body).<sup>14</sup> Pkh regulates chronological aging, cell size determination, and nutrient-induced growth resumption by phosphorylation and activation of the protein kinase Sch9.<sup>12,15</sup> Phosphorylation of Ypk by Pkh leads to the activation of its downstream signaling, which regulates endocytosis.<sup>16</sup> Also, through Ypk, Pkh is involved in the maintenance of the asymmetric distribution of aminophospholipids between the two leaflets of the plasma membrane and is involved in important aspects of the heat shock response.<sup>17</sup> Together, Pkh has been shown to have pleiotropic roles in yeast cells.

Genome sequencing revealed that *C. albicans* has two Pkh-related ORFs: orf19.5224, encoding a protein (CaPkh2) more similar to Pkh1 and Pkh2 and orf19.1196, which encodes a protein (CaPkh3) more similar to Pkh3. The role of Pkh from *C. albicans*, broadly assumed to be equivalent to the role in *S. cerevisiae*, has not been extensively investigated. However, biochemical and genetic research on *C. albicans* has shown important roles for the Pkh substrates CaPkc1, CaTpk1, and CaSch9. Deletion of *CaPKC1* results in cell lysis in hypotonic conditions and morphological aberrant cells, in agreement with an upstream role in the CWI pathway.<sup>18</sup> Also similarly to *S. cerevisiae*, CaTpk isoforms play important roles in cell growth, yeast to hypha transition,<sup>19,20</sup> stress response, and glycogen storage,<sup>21,22</sup> while CaSch9 is also required for morphogenesis and virulence.<sup>18,23</sup>

Most drug developments directed to protein kinases target the ATP-binding site. Due to the similarity of this site among

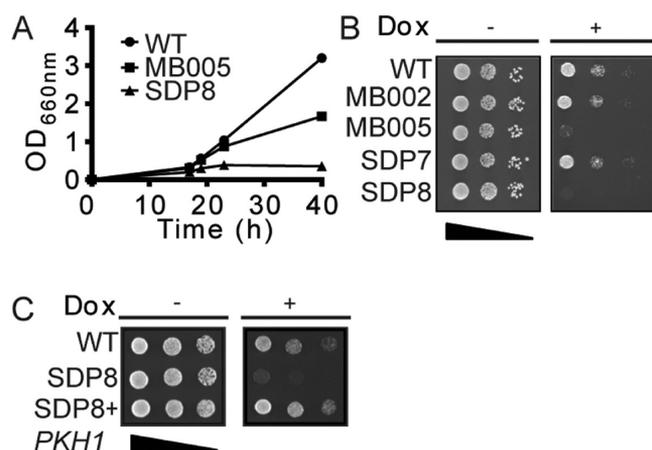
the protein kinases, such compounds are most often unspecific, inhibiting multiple protein kinases. Thus, compounds targeting the ATP-binding site of fungal Pkh are expected to also inhibit human PDK1 and multiple additional human kinases, producing unwanted side effects. Previous work identified protein kinase inhibitors that inhibited the CWI pathway and had antifungal activity against *C. albicans*.<sup>1</sup> The compounds (e.g., KP-372 and UCN-01) are known to inhibit several human protein kinases including PDK1 and may also target other kinases in yeasts. It has been speculated that higher selectivity will be identified by targeting sites different from the ATP-binding site on fungal Pkh.<sup>1</sup> In the present work, we further validate Pkh as an antifungal target and describe differences between the PIF-pocket of Pkh and its human ortholog PDK1. Notably, we describe a novel allosteric small compound, **PS77**, that binds to the PIF-pocket and preferentially inhibits CaPkh over PDK1. Together, our results open the field of drug development of antifungals based on the use of selective, allosteric protein kinase inhibitors.

## RESULTS AND DISCUSSION

It has been previously postulated that the PDK1 ortholog in fungal organisms, Pkh, could be a drug target. However, some questions still remained: what is the effect of Pkh deletion in yeasts in the absence of heat stress? Would Pkh inhibitors be cytostatic or cytotoxic? Would it be possible to develop antifungal kinase inhibitors with selectivity over human kinases? Answers to such questions could support the validation of Pkh as an antifungal drug target and the approach to achieve its selective inhibition in antifungal therapies. In the present work, we analyze the effects of Pkh depletion in yeast cells cultured in the absence of heat stress and further employed a multi-disciplinary approach, including biochemistry, structural biology, and chemical biology methods to answer the above questions.

**Effects Caused by Pkh Depletion in the Absence of Heat Stress.** Growth of yeast cells at stressful temperatures, in combination with a temperature-sensitive allele strategy, has been previously used to investigate the role of Pkh in *S. cerevisiae*. In order to investigate the effect of Pkh inhibition in the absence of heat stress, we prepared *pkh1Δ* and *pkh1Δ*

*pkh3Δ* yeast strains where *PKH2* was controlled by the doxycycline-repressible (*tetO<sub>7</sub>*) promoter (strains MB005 and SDP8; Table 1). Upon repression of *PKH2* transcription by the addition of doxycycline, MB005 cells continued to grow normally for about 20 h at which time the levels of *PKH2* mRNAs measured by RT-PCR decreased 9-fold, concomitant with a decrease in the number of dividing cells. Depletion of Pkh decreased the ability of yeasts to activate the CWI pathway (Supporting Information (SI) Figure 1). After 24 h of incubation with doxycycline, there was a 70% decrease in the number of budding cells of the MB005 strain. In line with this, the yeast growth in liquid media was severely affected in MB005 cells, with complete inhibition of growth in SDP8 cells (Figure 1A). Similarly, the strain depleted of Pkh1 and 2



**Figure 1.** *S. cerevisiae* cells lacking *PKH1* and having reduced expression of *PKH2* are not viable. (A) Suspension growth of the indicated yeast strains in the presence of 100 μg/mL doxycycline. (B–C) Serial 1:10 cell dilutions of the indicated strains spotted onto YPD agar plates in presence or absence of 100 μg/mL of doxycycline and incubated at 28 °C for 36 h. (C) Expression of *PKH1* from the pRS316 centromeric plasmid under the control of its own promoter reconstitutes growth of SDP8 in the presence of 100 μg/mL doxycycline.

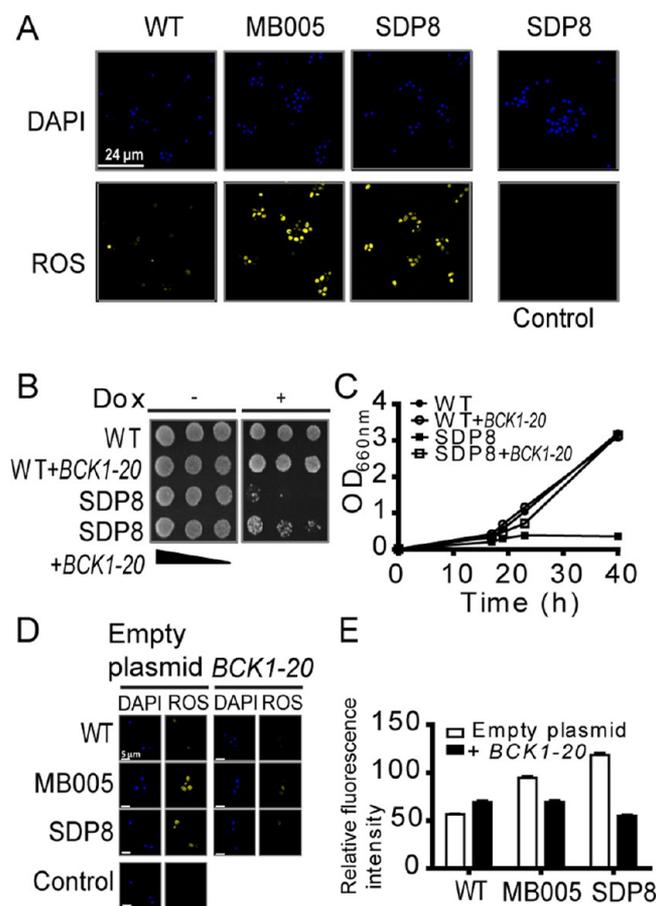
(MB005) and the strain depleted of the three Pkh isoforms (SDP8) had greatly reduced growth in solid media complemented with doxycycline, whereas strains still expressing normal levels of Pkh1 and Pkh3 (MB002) or Pkh1 (SDP7) were unaffected (Figure 1B). The growth defect in the presence of doxycycline was specifically caused by the depletion of the Pkh proteins, since growth of the SDP8 strain in doxycycline-containing media was reconstituted upon the expression of yeast *PKH1* (Figure 1C). The above data indicated that the yeast cells cannot adapt to growth, even in the absence of heat stress.

We note that our experimental model using the transcriptional repression of *PKH2* does not necessarily inform on the direct effects of the lack of Pkh. Certainly, a subset of the effects may be indirect and could depend on the compensatory mechanisms that may happen when the levels of Pkh are diminished. This is similar to the information that is routinely obtained in higher organisms by knockout or knock-down technologies. At any rate, the results clearly show that the depletion of Pkh is detrimental for the growth of *S. cerevisiae* cells when cultured in optimal conditions in liquid or in solid media and that the lack of Pkh cannot be compensated.

Global expression analyses further indicated that depletion of Pkh up-regulates genes involved in glycogen metabolism, protein folding, and response to oxidative stress (SI Results and SI Figure 2). The data were consistent with the hypothesis that long-term depletion of Pkh may cause oxidative stress, a feature that had not been previously recognized.

### Cells Depleted of Pkh Accumulate Reactive Oxygen Species (ROS).

To test the effect of Pkh depletion on oxidative stress, WT CML476, MB005, and SDP8 cells were treated with doxycycline for 24 h and then incubated with 1,2,3-dihydrorhodamine to monitor the generation of ROS. MB005 and SDP8 cells displayed more intense fluorescent staining than WT CML476 cells indicating higher presence of ROS (Figure 2A). Therefore, we conclude that the long-term depletion of Pkh produced increased levels of ROS in yeast cells. It has been shown that Pkh phosphorylates Pkc1 and that, in turn, Pkc1 phosphorylates and activates Bck1, the MAPKKK of the Slt2-MAPK pathway, thereby activating the CWI pathway. In WT CML476 and SDP8 cells, we then expressed



**Figure 2.** Depletion of Pkh activity induces oxidative stress in *S. cerevisiae*. (A) The depletion of Pkh under doxycycline produces the accumulation of ROS. (B–C) Expression of *BCK1-20* reconstitutes growth of the SDP8 strain in YPD agar (B) and in liquid media (C). (D–E) *BCK1-20* expression reverts the accumulation of ROS in the absence of Pkh. (E) Quantification of ROS by FAC sorting. (A and D) WT CML476 and its derivative strains, MB005 and SDP8, were treated with doxycycline for 24 h and loaded with dihydrorhodamine 123. The fluorescence of a sample of SDP8 cells not treated with DHR123 is also shown as a control (–DHR123). Nuclei were visualized by DAPI staining. Representative fields of the indicated strains are shown. Error bars denote the standard deviations.

the *BCK1-20* allele, which encodes for a constitutively active Bck1 kinase that renders an active Slr2.<sup>24</sup> Activation of the Slr2 pathway by the expression of *BCK1-20* partially rescued the lack of growth of SDP8 cells in the presence of doxycycline in both solid (Figure 2B) and liquid medium (Figure 2C), indicating that the lethality of Pkh-depleted cells was rescued, at least in part, by downstream activation of the CWI pathway.

We next investigated whether the oxidative stress detected in Pkh-depleted cells was also due to Pkc1 downstream effects. Quantification of ROS showed that both MB005 and SDP8 cells carrying the *BCK1-20* allele had a notably decreased quantity of ROS when compared with the same strains harboring the empty plasmid (Figure 2D,E). Taken together, these results indicate that depletion of Pkh causes an increase in ROS and that the downstream activation of the Slr2 MAP kinase pathway partially suppresses both the growth defect and the accumulation of ROS caused by the lack of Pkh.

**Long-Term Depletion of Pkh Induces Programmed Cell Death (PCD).** The presence of ROS could be a signal of apoptotic-like cell death in yeast. We hypothesized that the ROS detected in cells lacking Pkh proteins could be triggering a PCD process. One key feature of apoptosis is the fragmentation of the genomic DNA. Therefore, we examined the occurrence of double-strand DNA breaks in doxycycline-treated WT CML476 and MB005 cells using the TUNEL assay. Indeed, we observed that, after 24 h of incubation in the presence of doxycycline, MB005 cells displayed stronger staining for TUNEL than WT CML476 cells (Figure 3A). This signal of

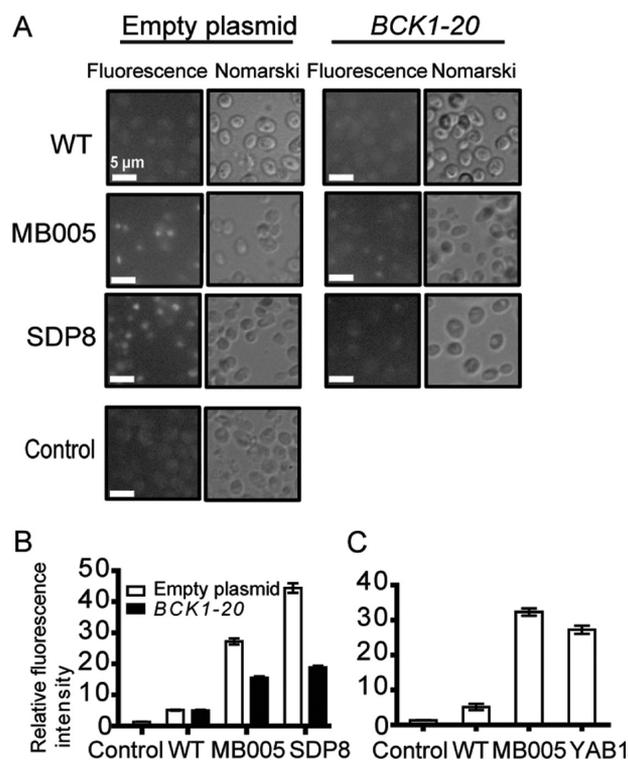
DNA fragmentation was even more pronounced in SDP8 cells. In both strains, the activation of the Slr2 MAP kinase pathway by the expression of the *BCK1-20* allele vastly decreased the number of double-strand breaks (Figure 3A,B). The above result is in agreement with the hypothesis that the long-term deletion of Pkh induces DNA fragmentation and that this effect is also mediated by the lack of Pkh-dependent activation of the Pkc1 downstream signaling cascade.

Some of the apoptotic stimuli in yeast, including treatment with H<sub>2</sub>O<sub>2</sub>,<sup>25</sup> require the yeast metacaspase Mca1 to trigger the apoptotic process, but other stimuli induce Mca1-independent apoptosis.<sup>26</sup> To investigate the possible role of the yeast metacaspase, we deleted the *MCA1* gene in the MB005 strain, generating the YAB100 strain (Table 1), and measured the DNA double-strand breaks after incubation with doxycycline for 24 h. MB005 cells displayed at least 6-fold more DNA double-strand breaks than WT CML476 cells. Additional disruption of *MCA1* still showed more than 5-fold the signal of WT CML476 cells (Figure 3C). Taken together, these results indicate that the apoptotic process triggered by the lack of Pkh is partially dependent on the activation of the Slr2 MAPK cascade but is not dependent on the Mca1 yeast metacaspase.

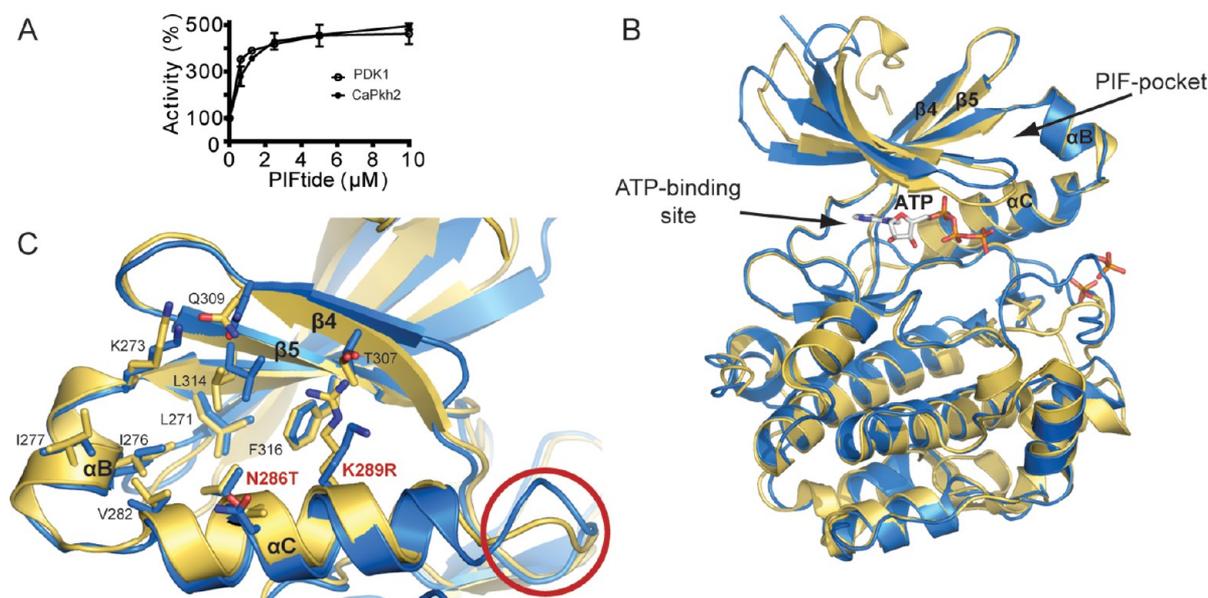
The above results indicate that yeast cells cannot cope with the long-term depletion of Pkh, even in optimal growth conditions, and that they die, displaying typical features of PCD. This effect supports the use of fungal Pkh as potential drug targets for antifungals, since one would predict that such drugs would be cytotoxic rather than cytostatic. Most importantly, the finding that depletion of Pkh is lethal and that it is also required for the response of yeasts to stresses suggests that selective drugs targeting fungal Pkh may be combined with other drugs producing cell wall stresses (e.g., echinocandins). While each drug would be effective on its own, we expect that the combination would synergize the effects of the drugs selectively in fungal organisms, by impeding the cellular responses to cell wall stresses, as very recently suggested for *Cryptococcus neoformans*.<sup>27</sup> Today, the combination of modern targeted drugs is a success in the treatment of HIV and HCV, and it is expected that future cancer therapies will involve the use of targeted drugs in personalized combinations. Our work highlights the immense potential for the combination of targeted drugs for the development of efficient antifungals. However, we should stress that the ideal antifungal or anti-infective drugs should have selectivity for the protein kinase from the infective organism over other protein kinases from the human host.

**Biochemical Similarities and Differences between CaPkh2 and PDK1.** *C. albicans* is an obligatory diploid organism, which contains two genes encoding putative orthologs of Pkh proteins (*CaPKH2* and *CaPKH3*). Heterozygous deletion of *CaPKH2* in the CAI4 *C. albicans* strain rendered viable cells. However, the heterozygous cells had a defect in glycogen accumulation, suggesting that the Pkh phosphorylation of Tpk1 and Tpk2, the catalytic subunits of PKA, mediating basic cellular processes including glycogen accumulation,<sup>21</sup> was sensitive to the haploinsufficiency of CaPkh2 (SI Figure 3). Interestingly, we were not able to obtain homozygous null yeast cells, indicating that CaPkh2 could be essential for the viability of this strain. This result reinforced the notion that CaPkh2 could also be essential for *C. albicans* and inhibitors of CaPkh2 could be used as antifungals.

Protein kinase inhibitors to be employed as anti-infectives in humans should have high selectivity and have preference for the



**Figure 3.** Depletion of Pkh causes DNA fragmentation. (A) Double-strand DNA breaks visualized by TUNEL staining. (B and C) Quantification of double-strand DNA breaks by FAC sorting. (B) *BCK1-20* expression reverts DNA fragmentation induced by depletion of Pkh. (C) DNA fragmentation induced by Pkh depletion is not reverted by deletion of the metacaspase gene *MCA1*. The control corresponds to the autofluorescence of MB005 cells containing the empty pRS316 plasmid. Error bars denote the standard deviations.



**Figure 4.** Biochemical and structural characterization of CaPkh2. (A) Activity assay showing that CaPkh2 is activated by PIFtide, similarly as PDK1. (B) Crystal structure of the catalytic domain of CaPkh2 (blue) superimposed on the structure of human PDK1 (yellow) (PDB code 1H1W). The structures are shown in cartoon representation; ATP and the phosphorylations on the activation loops are depicted as sticks. (C) Close-up view of the PIF-pocket with side chains constituting the pocket shown as sticks. The perspective in comparison to part B is rotated by  $\sim 180^\circ$  along a vertical axis. Although the PIF-pocket of CaPkh2 is structurally similar to the PIF-pocket of PDK1, essential residues differ (indicated with red labels), and one additional residue is inserted in the loop C-terminal to helix  $\alpha C$  (red circle).

pathogen over the human ortholog kinase to avoid side effects. To investigate the potential selectivity of the ATP-binding site and the allosteric regulatory PIF-pocket, we expressed GST-CaPkh2 in mammalian cells and N-terminally His-tagged CaPkh2 in insect cells. Both sources of purified CaPkh2 phosphorylated T308tide, a reference polypeptide used as a substrate of PDK1, with a specific activity of  $\sim 3 \mu\text{mol mg}^{-1} \text{min}^{-1}$ , which was about 2 fold higher than the specific activity of human PDK1 measured under identical conditions. Similarly to PDK1, CaPkh2 was activated  $\sim 3$ – $4$  fold by PIFtide (Figure 4A), a prototype 24 residue polypeptide that binds to the PIF-pocket and activates PDK1.<sup>4,28</sup> It was recently identified that the protein kinase inhibitor KP-372 inhibited the CWI pathway and had antifungal activity.<sup>1</sup> KP-372 was originally identified as a nonselective ATP-binding site inhibitor of the protein kinase PKB (also termed Akt)<sup>29</sup> and later found to inhibit PDK1 activity.<sup>30</sup> However, the direct inhibition of Pkh by KP-372 had not been verified *in vitro*. We therefore tested the relative ability of KP-372 to inhibit PDK1 and CaPkh2 in kinase assays using  $100 \mu\text{M}$  ATP, a relatively low concentration that favors binding of inhibitors to the ATP-binding site. Surprisingly, we observed that KP-372 failed to inhibit PDK1 and CaPkh2 *in vitro*, with 75% remaining activity at  $50 \mu\text{M}$ , whereas, as described, KP-372 indeed inhibited PKB/Akt (SI Figure 4A).

**Crystal Structure of CaPkh2.** To investigate the possible differences between PDK1 and CaPkh2 that could be targeted for the development of selective inhibitors, we attempted to crystallize full-length His-CaPkh2. We solved the crystal structure of CaPkh2 by molecular replacement using the PDK1 structure (PDB ID: 3HRC) and refined the CaPkh2 structure to  $3.16 \text{ \AA}$  resolution. The structure comprises only the catalytic domain (aa 237–510) of CaPkh2 (see SI Results). In the solved structure, the main chain and most side chains can be accurately traced, allowing the comparison of the structures of the catalytic domains of PDK1 and CaPkh2. The structure

shows the typical bilobal protein kinase structure, with the ATP-binding site in the cleft between both lobes (Figure 4B). The protein is phosphorylated at the activation loop, and the structure is in an overall active conformation, similar to the structures of PDK1 in crystal packing I<sup>31</sup> and crystal packing II.<sup>32</sup> However, similar to PDK1 structures in the absence of allosteric compounds, the active site is not in the fully closed-active conformation but in an intermediate conformation (Figure 4C), comparable to the structure of PKA in complex with staurosporine (PDB ID: 1STC). Moreover, the activation loop, which is seven residues longer than in human PDK1, adopts an unusual conformation that would sterically interfere with polypeptide substrate binding when compared to the structure of PKA in complex with PKI (PDB ID: 1ATP).

The ATP-binding site is almost identical between CaPkh2 and PDK1 (SI Results and SI Figure 5). In spite of the general similarities between PDK1 and CaPkh2, significant differences were observed at the allosteric PIF-pocket regulatory site. The PIF-pocket is located between the beta-strands  $\beta 4$  and  $\beta 5$  and helices  $\alpha B$  and  $\alpha C$ . Interestingly, CaPkh2 contains an additional residue in the loop following the regulatory helix  $\alpha C$  (Figure 4C). In addition, some key residues in the helix  $\alpha C$ , corresponding to Thr128 and Arg131 in PDK1, are replaced by Asn and Lys, respectively. Since these residues are constituents of the allosteric PIF-pocket binding site where small compounds bind,<sup>32,33</sup> they could well participate in selective interactions. Together, the crystal structure shows that while the ATP-binding site is almost identical between PDK1 and CaPkh2, the PIF-pocket may provide selectivity for the development of CaPkh2-selective allosteric small compounds.

Mutation of the residue at the center of the hydrophobic PIF-pocket in PDK1, Leu155, to Ser, Asp, or Glu renders proteins that have 3-fold higher specific activity.<sup>4</sup> In sharp contrast, the CaPkh2 mutant equivalent to Leu155Glu (CaPkh2 [Leu314Glu]) had vastly reduced specific activity

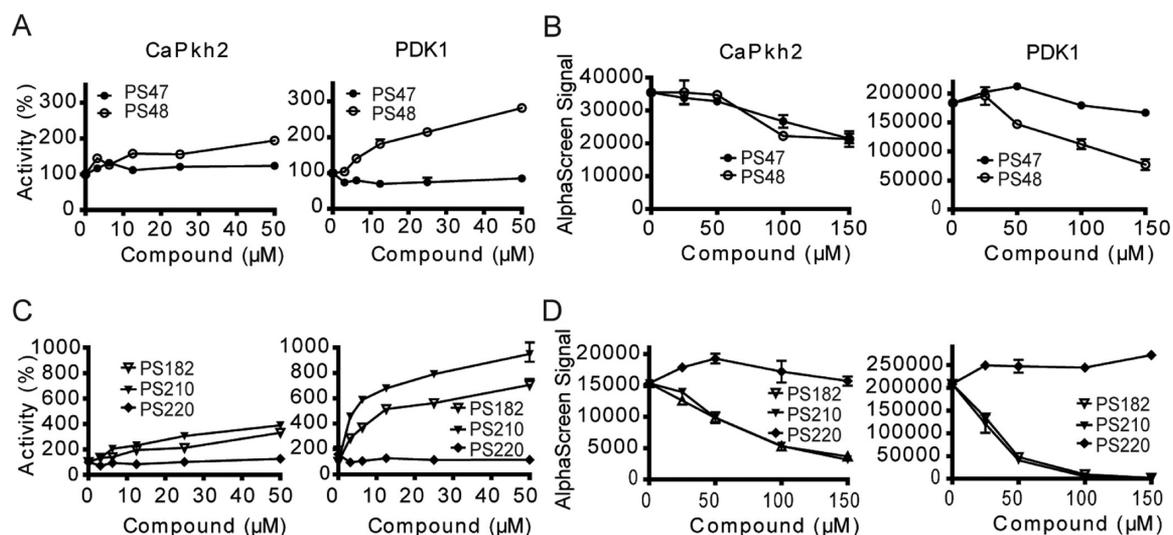
Table 2. Effect of Compounds on PDK1 and CaPkh2<sup>a</sup>

Compound name	Formula	CaPkh2					PDK1				
		Kinase activity			AlphaScreen		Kinase activity			AlphaScreen	
		Activity (%) 50 $\mu$ M	AC <sub>50</sub> ( $\mu$ M)	IC <sub>50</sub> ( $\mu$ M)	PIFtide bound (%) 100 $\mu$ M	EC <sub>50</sub> ( $\mu$ M)	Activity (%) 50 $\mu$ M	AC <sub>50</sub> ( $\mu$ M)	IC <sub>50</sub> ( $\mu$ M)	PIFtide bound (%) 100 $\mu$ M	EC <sub>50</sub> ( $\mu$ M)
PS76		96	-	n.e.	89	n.d.	45	-	n.d.	100	n.d.
PS77		3	-	17	5	44	37	-	38	91	n.d.
PS46		209	65	-	44	85	237	38 <sup>b</sup>	-	27	n.d.
PS47		123	n.e.	-	25	n.d.	80	n.e. <sup>c</sup>	-	2	n.d.
PS48		194	48	-	37	n.d.	281	8 <sup>c</sup>	-	39	97
PS182		331	n.d.	-	95	66	706	2 <sup>d</sup>	-	65	26
PS210		389	42	-	98	60	950	4 <sup>d</sup>	-	65	39
PS220		126	n.e.	-	100	n.e.	116	n.e.	-	100	n.e.

<sup>a</sup>n.d. = not determined; n.e. = no effect. <sup>b</sup>Ref 35. <sup>c</sup>Refs 32 and 38. <sup>d</sup>Refs 33 and 39.

(13% of the activity of wild type CaPkh2). Similarly, the mutation of Arg131 to Met renders a mutant PDK1 protein that has 2-fold higher specific activity<sup>34</sup> while the equivalent CaPkh2 mutant (Lys314Glu) had a slightly lower specific activity (63% of the activity of wild type CaPkh2). Together, the structural differences between the PIF-pockets of PDK1 and CaPkh2 and the additional differences in the activities of PDK1 and CaPkh2 proteins mutated at the PIF-pockets, provided a first indication that the PIF-pocket was structurally and functionally different between the human PDK1 and the *C. albicans* ortholog.

**Differences at the PIF-Pocket between PDK1 and CaPkh2.** We then tested small molecules from our PIF-pocket-directed focused library for their ability to affect the activity of CaPkh2 (Table 2). **PS48**, **PS210**, and **PS182** (Table 2) are *in vitro* activators of PDK1 that have been cocrystallized with PDK1,<sup>32,33</sup> confirming their binding to the PIF-pocket. Interestingly, **PS48**, **PS210**, and **PS182** had drastically decreased abilities to activate CaPkh2 (Figure 5A and C). In order to further characterize the interaction of small compounds with the PIF-pocket, we established AlphaScreen interaction-displacement assays using His-CaPkh2 or His-



**Figure 5.** Comparison of the PIF-pocket of CaPkh2 and PDK1 using small compounds allosteric activators that bind to the PIF-pocket of PDK1. (A) Effect of related compounds PS47 and PS48 on the activity of CaPkh2 and PDK1. (B) Displacement of PIFtide by PS47 and PS48. (C) Effect of related compounds PS182, PS210, and PS220 on the activity of CaPkh2 and PDK1. (D) Displacement of PIFtide by PS182, PS210, and PS220. The ability of compounds to displace the CaPkh2-PIFtide or PDK1-PIFtide interaction was evaluated using an AlphaScreen interaction–displacement assay. Error bars denote the standard deviations.

PDK1 and the biotinylated polypeptide PIFtide (SI Figure 6). Whereas inactive compounds (e.g., PS47 and PS220) did not displace the PDK1-PIFtide interaction, low-molecular-weight activators readily displaced the PDK1-PIFtide interaction. In comparison, PS48, PS210, and PS182 had decreased abilities to selectively displace the CaPkh2-PIFtide interaction (Figure 5B and D). Together, the data suggest that PS48, PS210, and PS182 had decreased abilities to bind to the PIF-pocket and decreased abilities to fully activate CaPkh2, providing further evidence that the PIF-pocket of CaPkh2 differed from that of PDK1.

**PS77 is a CaPkh2 PIF-Pocket Allosteric Inhibitor with Selectivity over PDK1.** Interestingly, we also identified that the compound 2-(3-(4-chlorophenyl)-1-(2-(4-chlorophenylthio)phenyl)-3-oxopropylthio) acetic acid (PS77; Table 2; see the synthesis and chemical characterization in the SI Data) inhibited CaPkh2 (Figure 6A). PS77 is a variant of PS46, which is a low-molecular-weight PDK1 activator (Table 2; compound 1 in ref 35). Biochemical and mutagenesis experiments showed that PS46 binds to the PIF-pocket,<sup>35</sup> a feature that was confirmed by the crystal structure of the PDK1-ATP-PS46 complex (Valerie Hindie, Pedro M. Alzari, and R.M.B., unpublished). Most interestingly, PS77 specifically inhibited CaPkh2 but had marginal inhibitory effect on PDK1 (Figure 6A). As a control for the specificity, the related compound 1-(4-chlorophenyl)-3-(2-(4-chlorophenylthio)phenyl)prop-2-en-1-one (PS76) did not inhibit CaPkh2. In complete agreement with the inhibition of CaPkh2, PS77 also displaced the interaction between His-CaPkh2 and biotin-PIFtide (Figure 6B) whereas PS76 had no effect on the displacement. In contrast, PS77 had vastly decreased ability to displace PIFtide from PDK1 (Figure 6B). Together, the results provided evidence that PS77 is a selective CaPkh2 allosteric inhibitor that competes with PIFtide for the binding to CaPkh2.

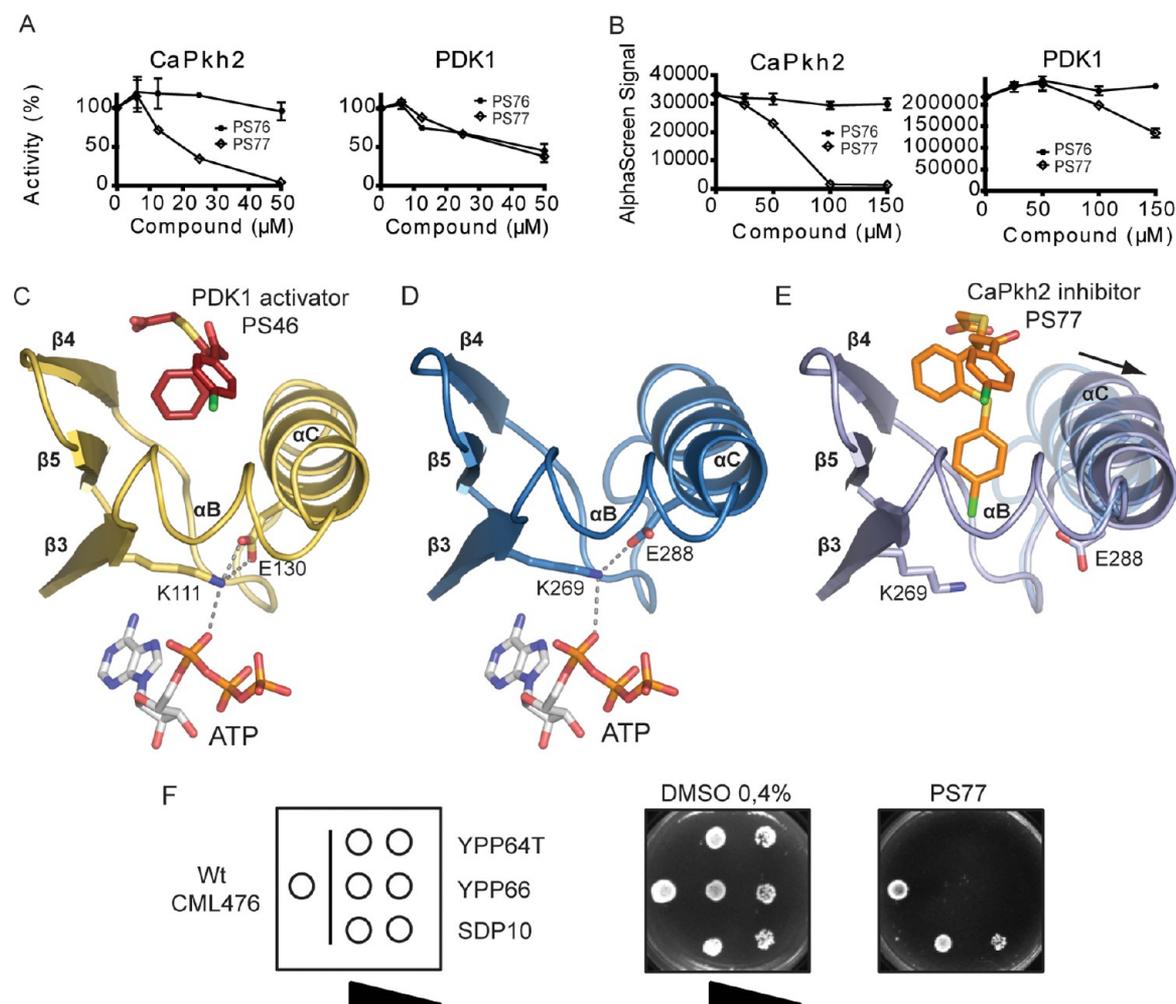
Since we have obtained the crystal structure of diverse allosteric activators and allosteric inhibitors bound to the PIF-pocket, it was of interest to model the binding of PS77 to the PIF-pocket of CaPkh2. Interestingly, PS77 does not fit into the

PIF-pocket in a manner equivalent to compound 1, PS48, or PS210 in the overall active structure of CaPkh2 or PDK1 since there is no apparent space for the diphenyl sulfide system. Since the helix  $\alpha C$  of AGC kinases in solution has a large degree of mobility, we envisage that the 2-phenylthio moiety of PS77 may occupy a novel pocket behind helix  $\alpha C$  (Figure 6C–E).

Together, the present work sheds light on the potential of CaPkh as an antifungal drug target, the potential to use Pkh inhibitors in combination with drugs producing cell wall stress to synergize effects in fungal infectious organisms, and further provides evidence that the PIF-pocket regulatory site on CaPkh provides the desired selectivity to distinguish CaPkh from PDK1 for selective antifungal drugs. In follow-up experiments we found that compound PS77 was not toxic to wild type *S. cerevisiae* cells or a yeast strain with deletion in *ERG6*, a mutation that often facilitates the permeability of small compounds into yeast cells. However, PS77 was toxic to yeast strains YPP66 and YPP64T ( $EC_{50}$  approximately 5  $\mu M$ ), that had deleted Pdr5, Snq2 and Yor1 ABC transporters and transcription factors Pdr1 and Pdr3 that regulate the expression of ABC transporters (Figure 6F and SI Figure 7A). This indicated that PS77 permeated yeast cells but that it needs to be improved to avoid interaction with yeast ABC transporters. In addition, we found that PS77, at higher concentrations (25  $\mu M$ ), was also toxic to mammalian cells in culture (SI Figure 7B) suggesting that more selective and potent compounds to CaPkh2 may be required to provide a therapeutic window. At any rate, PS77 represents a proof-of-principle that can be considered a starting point for drug development. Similar strategies targeting allosteric sites may be envisaged for other AGC kinases and other organisms producing fungal and parasitic infections.

## METHODS

General materials and methods, the details on the construction of mutated yeast strains, the microarray methods, the determination of Reactive Oxygen Species and DNA double-strand breaks, the *C. albicans* methods, the expression and



**Figure 6.** Novel compound PS77 is a selective allosteric inhibitor of CaPkh2. (A) PS77 selectively inhibits the kinase activity CaPkh2. (B) PS77 selectively displaces the interaction between CaPkh2 and PIFtide. Error bars denote the standard deviations. (C–E) Binding mode of PS46 and schematic representation of the possible binding mode of PS77 to the PIF-pocket of CaPkh2. (C) Binding of activator PS46 (shown as sticks with red carbon atoms) to human PDK1 (yellow cartoon representation). This compound stabilizes the active conformation where the salt-bridge network among Glu130, Lys 111, and the  $\alpha$ -phosphate of ATP is stabilized and primed for catalysis. (D) The crystal structure of CaPkh2 (blue cartoon representation) also revealed an active conformation and the highly conserved salt bridges. (E) Proposed binding mode of inhibitor PS77 to CaPkh2. Compound PS77 was docked manually into the PIF-pocket of CaPkh2 by (1) positioning the 3-(4-chlorophenyl) and 1-phenyl ring systems, similarly to the position of the two ring systems found in PS48, PS182, and PS210 crystal structures in complex with PDK1, and (2) allowing an opening movement of the helix  $\alpha$ -C (translated outward) and positioning the third ring system ((4-chlorophenyl)sulfanyl) in a newly formed deep tunnel, behind helix  $\alpha$ -C. For comparison, the original location of helix  $\alpha$ -C is shown in transparent blue. Consequently, the model shows that Glu288 is moved out of salt-bridging distance with Lys269 and is not hold in position any longer to interact with ATP, suggesting a possible mechanism for the inhibition of CaPkh2 kinase activity. (F) PS77 is toxic to YPP66 and YPP64T yeast strains mutated in ABC transporters. Wild type, SDP10, YPP66, and YPP64T yeast strains were spotted onto YPD agar plates in presence or absence of 50  $\mu$ M PS77 and incubated at 28  $^{\circ}$ C for 36 h. SDP10, YPP66, and YPP64T were spotted also at 1:10 dilution.

purification of protein kinases and the synthesis of PS77 are described in the Supporting Information (SI). The peptide used as substrate of PDK1 and Pkh2 was T308tide (KTFCGTPEYLAPEVRR), which is derived from the PDK1 phosphorylation site of PKB $\alpha$ /Akt1.<sup>4</sup> PIFtide (PDK1 Interacting Fragment peptide), which was characterized to bind to the PIF-pocket of PDK1,<sup>4</sup> has the sequence REPRILSEEE-QEMFRDFDYADWC. Biotin-PIFtide used in AlphaScreen assays has the sequence Biotin-REPRILSEEEQEMFRDFDYADWS.

**Yeast Strains and Culture Conditions.** The *S. cerevisiae* and *C. albicans* yeast strains used in this work are presented in Table 1 and SI Table 3, respectively. The yeast strains YPP66 and YPP64T were kindly provided by Hybrigenics Services

(<http://www.hybrigenics-services.com>). Additional information on the yeast cells is described in the Supporting Information.

**In Vitro Protein Kinase Activity Assays.** PDK1 and CaPkh2 activity assays were performed in a 96 well format essentially as previously described using T308tide as a substrate for PDK1.<sup>4,35</sup> Activity measurements were performed in duplicates or triplicates. Experiments were repeated at least twice. Additional information on the protein kinase activity assays is presented in the Supporting Information.

**Expression, Purification, and Crystallization of CaPkh2.** GST-CaPkh2 and GST-PDK1 were expressed in HEK293 cells using a transient transfection protocol and purified as previously described for GST-PDK1.<sup>4</sup> His-CaPkh2 was expressed in SF9 insect cells using a baculovirus expression

system and purified through consecutive Ni-NTA and gel filtration chromatography. His-CaPkh2 was concentrated to 5.6 mg mL<sup>-1</sup> and used for high throughput screening of crystallization conditions. Diffraction data were collected on BL14.1 operated by the Helmholtz-Zentrum Berlin (HZB) at the BESSY II electron storage ring. The crystal structure was solved by molecular replacement based on human PDK1 (PDB code 3HRC) and refined to 3.16 Å resolution. The details on the purification of GST-CaPkh2 and purification and crystallization of His-CaPkh2 are presented in the Supporting Information.

**His-CaPkh2 and His-PDK1 Interaction with Biotin-PIFtide and Displacement of the Interaction Using AlphaScreen Technology.** The AlphaScreen assay (Perkin-Elmer) was performed according to the manufacturer's protocol in 384-well microtiter plates. The His-CaPkh2 interaction with biotin-PIFtide was evaluated in a similar setup as previously performed with His-PDK1<sup>33</sup> but with variations in the order of addition of the beads and the incubation times. In brief, incubations were performed in white 384-well microtiter plates (Greiner) containing 20 μL of a reaction mix and 5 μL of beads. The reaction mix contained 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM dithiothreitol, 0.01% (v/v) Tween-20, 0.1% (w/v) BSA, 1 mM DMSO, 100 nM His-CaPkh2 or 100 nM His-PDK1, 50 nM Biotin-PIFtide, and the indicated concentrations of unlabeled PIFtide or small compounds. Subsequently, 5 μL of beads solution containing nickel chelate-coated acceptor beads and streptavidin-coated donor beads (20 μg/mL final concentrations) was added to the reaction mix. The reaction mix and the beads were incubated in the dark for 45 min at RT and the emission of light from the acceptor beads was measured in the EnVision reader (Perkin-Elmer) and analyzed using the GraphPad Prism software.

## ■ ASSOCIATED CONTENT

### Supporting Information

Additional figures and methods as described in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>

### Accession Codes

The coordinates and structure factors of the CaPkh2 crystal structure have been deposited in the RCSB Protein Data Bank (PDB ID 4C0T)

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### Author Contributions

D.P.-F. performed most of the experimental research in the paper including yeast genetics, microarray, protein purification, and biochemistry. A.C. was responsible for the yeast genetics and microarray studies and supervised D.P.-F., A.B., and J.F.-D.; M.E. was responsible for the synthesis of the library of compounds as part of different research projects; R.G. did the *C. albicans* experiments with S.P.; J.O.S. was responsible for the crystallography work and supervised D.P.-F. in the protein purification. R.M.B. was directly responsible for the biochemistry work and supervised D.P.-F. and E.S. R.M.B. was responsible for the overall project involving crystallography,

biochemistry, and synthetic and medicinal chemistry while A.C. was responsible for the yeast genetics and signaling part of the work. R.M.B. and A.C. wrote the manuscript with support from D.P.-F. and J.O.S.

## Notes

The authors declare no competing financial interest.

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