Fludioxonil Induces Drk1, a Fungal Group III Hybrid Histidine Kinase, To Dephosphorylate Its Downstream Target, Ypd1

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ABSTRACT Novel antifungal drugs and targets are urgently needed. Group III hybrid histidine kinases (HHKs) represent an appealing new therapeutic drug target because they are widely expressed in fungi but absent from humans. We investigated the mode of action of the widely utilized, effective fungicide fludioxonil. The drug acts in an HHK-dependent manner by constitutive activation of the HOG (high-osmolarity glycerol) pathway, but its mechanism of action is poorly understood. Here, we report a new mode of drug action that entails conversion of the HHK from a kinase into a phosphatase. We expressed Drk1 (dimorphism-regulating kinase), which is an intracellular group III HHK from the fungal pathogen *Blastomyces dermatitidis*, in *Saccharomyces cerevisiae*. Drk1 engendered drug sensitivity in *B. dermatitidis* and conferred sensitivity upon *S. cerevisiae*. In response to fludioxonil, Drk1 behaved as a phosphatase rather than as a kinase, leading to dephosphorylation of its downstream target, Ypd1, constitutive activation of the HOG pathway, and yeast cell death. Aspartic acid residue 1140 in the Drk1 receiver domain was required for *in vivo* phosphatase activity on Ypd1, and Hog1 was required for drug effect, indicating fidelity in HHK-dependent drug action. In *in vitro* assays with purified protein, intact Drk1 demonstrated intrinsic kinase activity, and the Drk1 receiver domain exhibited intrinsic phosphatase activity. However, fludioxonil failed to induce intact Drk1 to dephosphorylate Ypd1. We conclude that fludioxonil treatment *in vivo* likely acts on an upstream target that triggers HHK to become a phosphatase, which dephosphorylates its downstream target, Ypd1.

KEYWORDS antifungal mechanism, fungi, HOG signaling, kinase, antimicrobial agents

New antifungal and targets are urgently needed to combat the growing number of fungal infections that are diagnosed in vulnerable patients with compromised immunity, such as those receiving organ transplants and treatment for autoimmune diseases, or experiencing immune-suppressive disorders, such as AIDS (1–3). There are just four classes of antifungal drugs, and only one new class of drug, which targets cell wall glucan synthase, has come to market in the last decade (1, 4). Group III hybrid histidine kinases (HHKs) represent an understudied but appealing new antifungal therapeutic drug target, because they are not found in humans (5), they are conserved in pathogenic fungi (6), and they are required for the action of the effective agricultural fungicide fludioxonil (7–10). Phenylpyrrole drugs, including fludioxonil and fenpiclonil,

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FIG 1 Potential mechanisms of action for fludioxonil. (A) A representative HOG pathway in *S. cerevisiae* heterologously expressing Drk1. Under normal (nonstress) conditions, both Sln1 and Drk1 phosphorylate Ypd1, which in turn negatively regulates the downstream components of the HOG pathway. (B) If fludioxonil inhibits Drk1 kinase activity, Sln1 is still present to negatively regulate the pathway, preventing Hog1 phosphorylation. (C) If fludioxonil increases Drk1 kinase activity, this would further inhibit the HOG pathway, not activate it. (D) If fludioxonil alters Drk1's function, converting the kinase to phosphatase Ypd1 would be dephosphorylated instead of phosphorylated, which would constitutively activate the HOG pathway.

are derivatives of pyrrolnitrin, a natural antifungal product from *Pseudomonas pyrroncinia* (11, 12). These drugs are used widely on crops pre- and postharvest, as well as for seed treatment. Pyrrolnitrin acts by blocking mitochondrial respiration (13), and phenylpyrroles somehow interfere with the yeast osmoregulatory, or HOG (high-osmolarity glycerol), pathway (12). While group III HHKs and HOG pathway components are required for the fungicidal action of these compounds, the precise target and mode of action remain poorly understood.

The HOG pathway regulates the response to environmental stress in fungi (14, 15). This pathway has been extensively studied in *Saccharomyces cerevisiae* and enables the cell to adapt to stressors such as osmotic and oxidative stress. In fungi, HHKs such as Sln1 from *S. cerevisiae* negatively regulate this pathway (14, 16–19) (Fig. 1A). Under nonstress conditions, Sln1 autophosphorylates and transfers the phosphate to Ypd1, a histidine phosphotransfer protein. Ypd1 then transfers the phosphate to Ssk1, which blocks its interaction with its partners, Ssk2 and Ssk22. This block inhibits downstream components of the pathway, leaving Hog1 unphosphorylated. Upon hyperosmotic stress, Sln1 fails to autophosphorylate and Ssk1 becomes unphosphorylated, allowing it to interact with its downstream partners and thereby leading to Hog1 phosphorylation (19, 20). Phosphorylated Hog1 moves into the cell's nucleus, activating transcription factors and genes, several controlling glycerol production, which stabilizes the cell against osmotic stress (21–24).

HHKs have been studied in depth in fungi. There are 11 distinct groups of HHKs, classified according to their structure and function (16). In *S. cerevisiae*, Sln1 is a transmembrane group VI HHK. Although many fungi harbor multiple HHKs and nearly all have a group III HHK (6, 16), Sln1 is the only HHK in *S. cerevisiae*, and its deletion is lethal in this fungus (16, 25, 26). Surprisingly, HHKs in other classes, particularly group III HHKs, are able to complement this deletion (27, 28). Consequently, *S. cerevisiae* has been used as a model to study group III HHKs, for example, Os-1 in *Neurospora crassa* and Hik1 of *Magnaporthe grisea* (27, 29, 30). Because *S. cerevisiae* lacks a group III HHK and thus is resistant to fludioxonil, heterologous expression of a group III HHK in the

yeast engenders fludioxonil sensitivity (29, 31, 32). In this model, both the group III HHK and HOG pathway are required for the drug's action. Treatment causes constitutive Hog1 phosphorylation (32–34) and cell death, whereas deletion of pathway components engenders drug resistance (29, 31, 35). The drug's action also requires the N-terminal HAMP domain repeats that are a signature feature of group III HHKs (27, 36). Moreover, fungal crop pests that acquire resistance to fludioxonil or dicarboximides, another class of antifungals that also require group III HHKs, reveal mutations in these HAMP domains (9, 37, 38). These findings together have focused interest on the group III HHKs and HOG pathway in explaining fludioxonil's mode of action. However, the precise target and its effect on HHK function remain poorly understood.

An understudied function of histidine kinases involves phosphatase activity (39–41). DokA from *Dictyostelium* and LuxN from *Vibrio harveyi* have been shown to dephosphorylate their downstream phosphotransfer protein via the action of their receiver domain (39, 41). In *V. harveyi*, under conditions of low bacterial cell density, the hybrid sensor LuxN acts as a kinase, and under conditions of high density, in the presence of an autoinducer, the sensor acts as a phosphatase (41). To our knowledge, phosphatase activity in fungal group III HHKs has not been explored as a way to explain the mode of fludioxonil action on pathogenic fungi.

Most pathogenic fungi harbor group III HHKs (6), which should sensitize them to phenylpyrrole drugs such as fludioxonil. *Blastomyces dermatitidis*, the agent of blastomycosis, harbors the group III HHK Drk1 (dimorphism-regulating kinase) (6, 28). In the present study, we heterologously expressed Drk1 in *S. cerevisiae* to study the mode of action of fludioxonil. Furukawa et al. (31) previously expressed the group III HHK Nik1 from *Debaryomyces hansenii* (DhNik1) in *S. cerevisiae* and found that fludioxonil treatment impaired Ypd1 signaling. Here, we tested whether fludioxonil treatment converts group III HHKs from a kinase into a phosphatase, prompting dephosphorylation of Ypd1 and constitutive activation of the HOG pathway.

Here, we furnish direct evidence that Drk1 induces dephosphorylation of Ypd1 in response to fludioxonil *in vivo*. We also show that the Drk1 receiver domain has an intrinsic ability to dephosphorylate Ypd1 *in vitro* when untethered from the kinase domain. Intact Drk1 protein, while active as a kinase *in vitro*, fails to show phosphatase activity *in vitro* in response to fludioxonil, arguing that the drug acts through an intermediate *in vivo*.

RESULTS

Drk1 functions as a traditional group III hybrid histidine kinase. Because most fungal pathogens of humans contain a group III HHK (6), we first investigated the activity of fludioxonil against six common fungal pathogens. Using a disc diffusion assay, we found that fludioxonil was able to kill all of the fungi tested, as indicated by zones of inhibition (Fig. 2A). *B. dermatitidis* was extremely sensitive and the zone of inhibition occupied nearly the entire well, whereas *Candida albicans* was one of the least sensitive and had the smallest zone of inhibition, even at an increased concentration of fludioxonil. Since *C. albicans* ATCC strain 18804 showed a small zone of inhibition in the disc diffusion assay and strains vary in their susceptibility (42), we verified group III HHK-dependent sensitivity to fludioxonil using the more sensitive K1 strain either sufficient or deficient in Nik1, the group III HHK in *C. albicans*. At 1.25 $\mu\text{g/ml}$ fludioxonil, growth of the wild-type strain (Nik1^{+/+}) was inhibited over 80%, whereas the Nik1^{-/-} strain was only inhibited 5%. The single-allele Nik1 strain (Nik1^{-/+}) revealed an intermediate phenotype (Fig. 2B). Thus, fludioxonil is active against all of the human pathogens tested.

In view of the sensitivity of *B. dermatitidis* to fludioxonil, we sought to ensure that the group III HHK Drk1 is necessary for the drug's effect. To do so, we silenced Drk1 using RNA interference (RNAi) and tested the yeast's sensitivity to fludioxonil in liquid culture (Fig. 2C). The unsilenced control grew well in medium containing the vehicle alone but failed to grow in the presence of fludioxonil. The Drk1 RNAi strain, however, grew equally well in both the vehicle- and fludioxonil-containing media. Thus, fludio-

FIG 2 Sensitivity to fludioxonil among fungal pathogens, including *Blastomyces dermatitidis*. (A) Various fungal pathogens of humans are sensitive to fludioxonil, as seen in a disc diffusion assay. (B) Growth of *C. albicans* Nik1^{+/+}, Nik1^{+/-}, and Nik1^{-/-} in 1.25 μ g/ml fludioxonil, normalized to growth in DMSO alone. Error bars represent the means $\pm$ standard errors of the means (SEM) from four experimental replicates. (C) Wild-type (WT) or Drk1 RNAi strains of *B. dermatitidis* were grown in the presence or absence of 1 μ g/ml fludioxonil for 3 days at 37°C. (D) Hog1 phosphorylation of WT or Drk1 RNAi strains of *B. dermatitidis* exposed to 1 μ g/ml fludioxonil for the indicated times. (E) RT-PCR analysis of *B. dermatitidis* Gpd1 (*BdGpd1*) Gpd1, *BdXyl2*, *BdArDH*, and *BdCut1* in WT or Drk1 RNAi strains of *B. dermatitidis* exposed to 1 μ g/ml fludioxonil for 90 min. Data are normalized to alpha tubulin and gene expression at 0 min and are represented as means $\pm$ SEM.

oxonil sensitivity in *B. dermatitidis* requires Drk1. Since fludioxonil constitutively activates the HOG pathway, we tested whether Drk1-dependent sensitivity requires HOG signaling, as measured by phosphorylation of Hog1 (33, 34). Hog1 became phosphorylated in response to 1 μ g/ml fludioxonil in wild-type *B. dermatitidis* but not in the Drk1 RNAi strain (Fig. 2D). The Drk1 RNAi strain was still able to respond to NaCl stress by Hog1 phosphorylation (data not shown). Thus, Drk1 is required for HOG pathway signaling in response to fludioxonil but not salt stress. To finally establish the role of Drk1 in fludioxonil sensitivity in *B. dermatitidis*, we examined downstream osmoticants that are known to stabilize growth under osmotic stress, e.g., genes that are potentially involved in the response to fludioxonil and osmotic stress, such as *B. dermatitidis*

FIG 3 *Saccharomyces cerevisiae* as a model to study group III HHK-mediated sensitivity to fludioxonil. (A and B) *Saccharomyces cerevisiae* cultures were grown for 48 h in a round-bottom 96-well plate at 30°C in the presence of either DMSO (control) or 25 μ g/ml fludioxonil in duplicate. Cell growth is evidenced by accumulation at the bottom of the well. Note that the white arc at the top of the wells is the light from the scanner. (A) WT *S. cerevisiae* (strain BY4741) and *S. cerevisiae* expressing either unmutated Drk1 or Drk1 D1140N, both under the control of a galactose-inducible promoter. WT *S. cerevisiae* does not express Drk1 in either glucose or galactose, but Drk1 and Drk1 D1140N *S. cerevisiae* express their respective forms of Drk1 only in the presence of galactose. (B) WT *S. cerevisiae* (strain W303-1A) and isogenic *S. cerevisiae* Δ Hog1 either alone or transformed with galactose-inducible Drk1 were used. WT and Δ Hog1 strains do not express Drk1 under any condition, and the Drk1-transformed strains only express Drk1 when grown in galactose. (C) Western blot of phosphorylated Hog1 and total Hog1 in WT *S. cerevisiae* (strain BY4741) transformed with either empty vector or galactose-inducible Drk1 exposed to either 0.4 M NaCl or 25 μ g/ml fludioxonil.

glycerol-3-phosphate dehydrogenase (*BdGpd1*), xylitol dehydrogenase (*BdXyl2*), L-arabitol dehydrogenase (*BdArDH*), and phosphatidyl synthase (*BdCut1*) (34, 43–46). After 90 min of exposure to fludioxonil, the level of transcripts of each of the genes rose substantially in wild-type *B. dermatitidis* compared to the RNAi strain (Fig. 2E). Thus, Drk1 also is required for the yeast's transcriptional response to fludioxonil. Taken together, these data establish that Drk1 behaves as a prototypical group III HHK in mediating HOG signaling and downstream events in response to fludioxonil.

***S. cerevisiae* as a model to study Drk1's response to fludioxonil.** We exploited *S. cerevisiae* as a model to study the action of fludioxonil, since it lacks group III HHKs and therefore is intrinsically resistant to the drug (29, 30). We expressed Drk1 heterologously in *S. cerevisiae* rather than study Drk1 endogenously in *B. dermatitidis* due to the tractability of *S. cerevisiae* and the extensive base of knowledge and tools available to investigate HOG signaling therein. We first validated that Drk1 shows fidelity with group III HHKs in rendering *S. cerevisiae* sensitive to fludioxonil. We utilized a galactose-inducible promoter to selectively express Drk1 (Fig. 3A) and grew Drk1-expressing *S. cerevisiae* in glucose or galactose, in either solvent or fludioxonil. Fludioxonil killed the

yeast only when Drk1 was expressed. Because fludioxonil sensitivity is known to require the conserved aspartate residue at position 1140 in the receiver domain (29, 30), we mutated the aspartate to asparagine to yield Drk1 D1140N. As expected, this mutation in Drk1 engendered fludioxonil resistance, as evidenced by yeast accumulation at the bottom of the well (Fig. 3A). We also sought to confirm that Drk1-dependent drug sensitivity requires Hog1. We used a Hog1 deletion strain of *S. cerevisiae* that expressed Drk1 and treated it with fludioxonil. In the absence of Hog1, Drk1-expressing yeast demonstrated resistance to fludioxonil (Fig. 3B). Finally, we investigated whether Hog1 became phosphorylated in response to fludioxonil in Drk1-expressing *S. cerevisiae*. Hog1 rapidly became phosphorylated in response to 0.4 M NaCl in both strains, whereas only the Drk1-expressing yeast showed Hog1 phosphorylation in response to fludioxonil (Fig. 3C). Taken together, these results establish that Drk1-expressing *S. cerevisiae* is a robust model for studying group III HHK-mediated sensitivity to fludioxonil in a manner that requires HOG pathway signaling.

Ypd1 is dephosphorylated *in vivo* in response to fludioxonil. We envision three ways in which fludioxonil may act on Drk1: inhibition, activation, or changing its function (Fig. 1B to D). It is unlikely fludioxonil inhibits Drk1, since inhibition would be similar to not expressing Drk1, and yeast, like *S. cerevisiae*, that lack such HHKs are resistant to fludioxonil. Likewise, fludioxonil likely does not increase Drk1 activity. Since group III HHKs are thought to negatively regulate the HOG pathway, increased activity would further inhibit the pathway, not activate it. That leaves the last possibility, which is that fludioxonil alters the function of group III HHKs such as Drk1. We specifically hypothesize that fludioxonil induces Drk1 to alter its function from kinase to phosphatase activity, thereby dephosphorylating Ypd1, which would lead to constitutive activation of the HOG pathway and cell death.

To test our hypothesis that Drk1 dephosphorylates Ypd1 in the presence of fludioxonil, we used an *S. cerevisiae* model to monitor Ypd1 phosphorylation *in vivo*. We exposed Drk1- and GST-Ypd1-expressing *S. cerevisiae* to NaCl (positive control) or fludioxonil and purified Ypd1 via the glutathione *S*-transferase (GST) tag. Phosphorimaging showed sharply reduced phosphorylation from both treatments (Fig. 4A and B). NaCl exposure of the yeast reduced Ypd1 phosphorylation by 43%, and fludioxonil exposure reduced Ypd1 phosphorylation by 97%. Coomassie staining of the gel following phosphorimaging confirmed roughly equal protein loading between the samples. Thus, in yeast that express Drk1, Ypd1 becomes dephosphorylated *in vivo* in response to salt stress, as would be expected, but also in response to treatment with fludioxonil.

Since Drk1 is necessary for fludioxonil sensitivity, we validated that dephosphorylation of Ypd1 in response to fludioxonil is Drk1 dependent. Here, we expressed Drk1 conditionally in galactose (Drk1 induction) or glucose (no induction) and quantified Ypd1 phosphorylation in response to fludioxonil. Ypd1 phosphorylation was diminished in response to fludioxonil in the Drk1-induced sample but not in the uninduced sample (Fig. 4C and D). In the Drk1-induced sample, Ypd1 phosphorylation was decreased by 96%, whereas it was reduced by only 7% in the Drk1-uninduced sample. We noted that the overall amount of Ypd1 phosphorylation in the Drk1-uninduced sample was lower than that in the Drk1-induced sample. This is likely due to the fact that Ypd1 is phosphorylated by only Sln1 in the Drk1-uninduced yeast and by both Sln1 and Drk1 in the Drk1-induced yeast. From these data taken together, we conclude that dephosphorylation of Ypd1 in the yeast in response to fludioxonil is Drk1 dependent.

When the Drk1 active aspartate residue 1140 is mutated, fludioxonil sensitivity is abolished (Fig. 3A). We investigated whether mutating this residue prevents Drk1-mediated dephosphorylation of Ypd1 in response to fludioxonil. Compared to the unmutated Drk1, Drk1 D1140N-expressing yeast did not show a decrease in Ypd1 phosphorylation in the presence of fludioxonil (Fig. 4E and F). After fludioxonil exposure, Ypd1 phosphorylation in the intact Drk1-expressing *S. cerevisiae* decreased by 92%, whereas Ypd1 phosphorylation actually increased slightly in the Drk1 D1140N-

FIG 4 Fludioxonil induces Drk1-dependent Ypd1 dephosphorylation *in vivo*. *S. cerevisiae* labeled with [32 P]orthophosphate was exposed to either 0.4 M NaCl for 1 min or 25 μ g/ml fludioxonil for 15 min. GST(S93A)-Ypd1 was purified from cell lysate using glutathione Sepharose resin, and samples were run on an SDS-PAGE gel followed by phosphorimaging (left) and quantification of signal intensity via densitometry (right). Gels were also Coomassie stained for total protein. (Right) Ypd1 phosphorylation is represented as the percentage of the signal relative to the starting signal. 32 P and Coomassie images are representative of two (A to D) or three (E and F) independent experiments. Signal quantification data are the means $\pm$ SEM from two (A to D) or three (E and F) independent experiments. (A and B) GST (S93A)-Ypd1 *S. cerevisiae* transformed with galactose-inducible Drk1. (C and D) GST (S93A)-Ypd1 *S. cerevisiae* transformed with galactose-inducible Drk1 grown in either glucose or galactose. The uninduced cultures (no Drk1 expression) were grown in glucose, and the induced cultures (Drk1 expression) were grown in galactose. (E and F) GST (S93A)-Ypd1 *S. cerevisiae* transformed with either galactose-inducible Drk1 or Drk1 D1140N was used.

expressing yeast. The Drk1 D1140N samples revealed an overall decrease in Ypd1 phosphorylation similar to the Drk1-uninduced yeast described above. Since the active aspartate is required to transfer the phosphate to Ypd1 (18), the Drk1 D1140N samples rely only on Sln1 to phosphorylate Ypd1, whereas the unmutated Drk1 samples have both Sln1 and Drk1 for this function. These data together establish that intact, wild-type Drk1 is required to dephosphorylate Ypd1 in response to fludioxonil.

The Drk1 receiver domain (Drk1R) displays intrinsic phosphatase activity, dephosphorylating Ypd1 independent of fludioxonil. The receiver domain of the

FIG 5 Drk1 receiver domain (Drk1R) harbors intrinsic phosphatase activity *in vitro* and dephosphorylates Ypd1 independent of fludioxonil. Phosphorylated resin-bound GST-Ypd1 was obtained by incubation with Sln1K, Sln1R, and [γ - 32 P]ATP followed by purification from the other components. The sample was divided and Drk1R (Drk1 receiver) or Drk1R D1140N was added (as indicated), and replicate samples were taken at the indicated time points. Samples were run on an SDS-PAGE gel followed by phosphorimaging and quantification of signal intensity via densitometry. 32 P and signal quantification are representative of two (A and B) or three (C and D) independent experiments. Signal quantification data are the means $\pm$ SEM from the two experimental replicates. (A and B) Ypd1 dephosphorylation in the presence or absence of Drk1R. Arrows indicate the location of Ypd1 and Drk1R. (C and D) Ypd1 dephosphorylation after incubation with either Drk1R or Drk1R D1140N. Note that Drk1R incorporates 32 P signal but Drk1R D1140N does not.

bacterial hybrid histidine kinase LuxN dephosphorylates its downstream target when the activity of the kinase domain is blocked (41). We sought to determine whether the receiver domain of the group III HHK Drk1 (Drk1R) similarly harbors phosphatase activity and can dephosphorylate Ypd1. We expressed and purified recombinant Drk1R (amino acids 1084 to 1288) and incubated it *in vitro* with 32 P-tagged Ypd1. Ypd1 was dephosphorylated by Drk1R in a time-dependent manner with reduction by nearly 75% after 60 min of incubation, whereas no reduction was observed in the absence of Drk1R (Fig. 5A and B). In LuxN, the conserved aspartate residue is required for the receiver to dephosphorylate its downstream target (41). We tested the role of the conserved aspartate in Drk1R for mediating phosphatase activity *in vitro* by expressing and purifying Drk1R D1140N. Here, we incubated either wild-type or mutated Drk1R with 32 P-tagged Ypd1. Drk1R D1140N showed blunted kinetics compared to those of wild-type Drk1R in the ability to dephosphorylate Ypd1, but it still dephosphorylated Ypd1 to nearly the same extent by the end of the 60-min incubation (Fig. 5C and D). We did observe that unmutated Drk1R itself became radiolabeled when incubated with 32 P-Ypd1, but that signal was absent with Drk1R D1140N. These data taken together reveal that Drk1R has an intrinsic ability to dephosphorylate Ypd1, but its action may not fully require the conserved aspartate residue.

FIG 6 Fludioxonil fails to induce intact, full-length Drk1 to dephosphorylate Ypd1 *in vitro*. (A) Purified resin-bound Drk1 (full length) is active in an *in vitro* kinase assay. Drk1 autophosphorylates and also transfers its phosphate to Ypd1. (B) Phosphorylated resin-bound GST-Ypd1 was obtained by incubation with Sln1K (kinase domain), Sln1R (receiver domain), and [γ - 32 P]ATP, followed by purification from the other components. Phosphorylated Ypd1 was added to Drk1, and either 2% DMSO or 2 mg/ml fludioxonil was added. Replicate samples were taken at the indicated time points. Samples were run on an SDS-PAGE gel followed by phosphorimaging and quantification of signal intensity via densitometry. 32 P and signal quantification are representative of at least five independent experiments. Signal quantification data are the means $\pm$ SEM from two experimental replicates.

Fludioxonil does not stimulate full-length Drk1 to dephosphorylate Ypd1 *in vitro*. Our findings so far indicate that fludioxonil induces Drk1 to dephosphorylate Ypd1 *in vivo*, and that the receiver domain of Drk1 (Drk1R) harbors intrinsic phosphatase activity *in vitro* in the above-described dephosphorylation assay. We next sought to test whether intact, full-length Drk1 also dephosphorylates Ypd1 *in vitro* or could be stimulated to do so by exposure to fludioxonil, e.g., by testing whether the drug acts directly on the group III HHK to induce its phosphatase activity. We first verified that purified intact Drk1 protein was functional. To do so, we employed a traditional HHK kinase assay (47) to assess *in vitro* kinase activity of Drk1. We observed autophosphorylation of Drk1 and subsequent phosphotransfer onto Ypd1 (Fig. 6A), indicating that Drk1 was both functional and capable of interacting with Ypd1. The latter result was especially important because both Drk1 and Ypd1 are resin bound in the above-described *in vitro* assay, so there could be steric hindrance preventing interaction between the two proteins. Having established that intact Drk1 displays proper kinase

activity *in vitro*, we tested whether the protein displays intrinsic phosphatase activity *in vitro* or whether it does so in response to treatment with fludioxonil, as it does *in vivo*. Intact Drk1 failed to exhibit phosphatase activity upon Ypd1 either in the absence (dimethyl sulfoxide [DMSO] solvent control) or the presence of fludioxonil (Fig. 6B and C). Thus, Ypd1 phosphorylation was unchanged under either condition. This difference of the *in vivo* and *in vitro* response of intact Drk1 to fludioxonil, together with the *in vitro* evidence that Drk1R harbors intrinsic phosphatase activity in this assay, strongly argues that fludioxonil acts on an intermediate to induce the conversion of Drk1 from a kinase to a phosphatase.

DISCUSSION

Fludioxonil is a widely used, potent agricultural fungicide, but its mode of action remains ill defined (12). We show here that the drug is active against a number of fungal pathogens of humans, underscoring the significance of understanding the drug's target and mode of action. Fludioxonil action has been shown to require group III HHKs and downstream elements of the HOG signaling pathway, but the mechanism remains unclear. In this study, we sought to elucidate the effects of fludioxonil on the activity of the prototypical group III HHK Drk1, derived from the human fungal pathogen *B. dermatitidis*. We demonstrated that Ypd1 phosphorylation is decreased *in vivo* in response to fludioxonil. While others have inferred altered Ypd1 signaling using a *LacZ* reporter system (31), our results directly demonstrate altered phosphorylation status of Ypd1 through reduced ^{32}P signal, extending those findings. We show that Ypd1 is dephosphorylated specifically in response to fludioxonil only when Drk1 is present and functional and not when *S. cerevisiae* is resistant to fludioxonil, e.g., if Drk1 is not expressed or if aspartate D1140 is mutated. Our results indicate that fludioxonil induces Drk1 to dephosphorylate Ypd1 and that this activity requires the conserved aspartate. However, it is also formally possible that Drk1 merely binds Ypd1, preventing it from interacting with and becoming phosphorylated by Sln1.

Because of the dependence of Drk1-engendered drug sensitivity on the conserved aspartate, we surmised that the dephosphorylation of Ypd1 requires the receiver domain of Drk1. In the case of LuxN from *V. harveyi*, Freeman et al. (41) observed that if the kinase activity was blocked constitutively through a mutation, the receiver domain would dephosphorylate its downstream protein target. They called this result "phosphatase activity." Others have opined that this is "signal decay," i.e., the phosphate traveling in a backwards direction, instead of true phosphatase activity (48). Since this dephosphorylation by LuxN in response to autoinducer 1 required the conserved aspartate, as with Drk1 and fludioxonil, we hypothesized that Drk1 likewise acquired phosphatase activity in response to drug exposure. Similar to the LuxN receiver domain, which harbors an intrinsic ability to dephosphorylate its downstream target, the Drk1 receiver domain likewise harbors intrinsic phosphatase activity; it dephosphorylates Ypd1 *in vitro* when expressed separately from the kinase domain. This intrinsic activity of the receiver depended in part on aspartate 1140, but not to the same extent as with LuxN. While we observed differences between wild-type Drk1R and Drk1R D1140N in the kinetics of dephosphorylation of Ypd1, mutated Drk1R was still able to dephosphorylate Ypd1. Thus, unlike in LuxN, the conserved aspartate in Drk1 was not absolutely required for phosphatase function of the receiver domain, since the extent of Ypd1 dephosphorylation was ultimately similar between mutated and unmutated Drk1R after 60 min. It is possible that if the aspartate is present in Drk1R it can accept a phosphate from Ypd1, leading to a signal decay mechanism. However, if the aspartate is mutated, Drk1R D1140N has a distinct mechanism by which it dephosphorylates Ypd1. We cannot exclude that a change in protein folding of D1140N accounts for the kinetic difference observed. While we use the term "phosphatase" to describe the dephosphorylation of Ypd1 by Drk1, we have not formally distinguished between phosphatase activity and signal decay mediated by Drk1.

Freeman et al. (41) proposed that the LuxN receiver domain is intrinsically able to dephosphorylate its downstream target LuxU, but that that activity could be dominated

by the kinase activity. Thus, only when the kinase is blocked is the dephosphorylation able to occur. Furukawa et al. (31) also reported that when the group III HHK Nik1 is mutated (by deleting the first four HAMP domains) such that it acquires constitutive kinase activity, it fails to respond to fludioxonil. Since we find that the Drk1 receiver domain can dephosphorylate Ypd1 when untethered from the kinase domain, it is possible that fludioxonil's mechanism of action also depends upon modulation of Drk1 kinase activity, allowing for the intrinsic ability of the receiver domain to emerge and dephosphorylate Ypd1.

We investigated whether fludioxonil acts directly on Drk1 to alter its function. Since we found that the Drk1 receiver domain evinced phosphatase activity on Ypd1 in our *in vitro* assay, we exploited this assay to test direct drug action on intact Drk1. One prevailing view is that fludioxonil directly targets HHKs through interaction with the HAMP domains (27, 31, 32). Deletion of selected HHK HAMP domains can render yeast resistant to fludioxonil (32). We found no evidence that fludioxonil directly induces the action of intact Drk1, e.g., the drug failed to induce dephosphorylation of Ypd1 by intact Drk1 *in vitro*. We excluded caveats that might explain our results. The first is that recombinant intact Drk1 may not be functional *in vitro*. This is unlikely, since we demonstrated Drk1 autophosphorylation and phosphotransfer from purified intact Drk1 to Ypd1. The second is that the buffer reaction conditions do not permit dephosphorylation of Ypd1. This also seems unlikely, since these buffer conditions were amenable for Drk1R to dephosphorylate Ypd1 independent of fludioxonil exposure. More likely than these technical caveats is the possibility that Drk1 is not a direct target of fludioxonil but instead requires an intermediate to signal to Drk1.

Others have found non-group III HHK enzymes that are affected by fludioxonil *in vitro*. For example, Karadag and Ozhan (49) found that fludioxonil inhibits bovine liver catalase activity and Karadag and Bilgin (50) saw that human superoxide dismutase (SOD) activity was also decreased in response to fludioxonil. It is possible that one of these enzymes, or another unidentified target, is directly affected by fludioxonil *in vivo* and in turn modulates the intracellular environment. Drk1 could then sense and respond to that change. Figure 7 shows our hypothesized model of how fludioxonil affects Drk1. This mechanism does not negate other reported findings regarding group III HHKs and fludioxonil. Since Drk1 is thought to be a sensor for the HOG pathway, which can respond to both osmotic and oxidative stress, perhaps Drk1 is signaled by an oxidative change through its HAMP domains, which in turn modulates its kinase activity, allowing Drk1 to acquire phosphatase activity and dephosphorylate Ypd1. More work must be done to determine the exact stimulus that Drk1 senses, allowing it to convert from kinase to phosphatase in response to fludioxonil. The extent to which HHKs can be induced to convert from a kinase to a phosphatase makes them potentially druggable and appealing targets.

MATERIALS AND METHODS

Strains, vectors, and growth conditions. Strains and vectors used in this study are listed in Tables 1 and 2, respectively. *S. cerevisiae* was grown in synthetic complete (SC) medium (58) lacking uracil and/or histidine with either 2% glucose, 2% galactose plus 1% raffinose (induction media), or 2% raffinose (overnight growth) as the carbon source. *S. cerevisiae* cells were grown at 30°C either stationary (96-well plate assays) or shaking at 200 rpm in Erlenmeyer flasks. *C. albicans* was maintained on yeast peptone dextrose (YPD) at 30°C. *Cryptococcus* spp., *Aspergillus fumigatus*, and *Rhizopus oryzae* were grown on YPD at 37°C. *B. dermatitidis* was grown on *Histoplasma* macrophage medium (HMM) (59) at 37°C.

Cloning and strain construction. Primer sequences used for vector construction are listed in Table 3. The vectors constructed were as follows.

pDONR-Drk1. Drk1 was first amplified from *B. dermatitidis* 26199 cDNA using Elongase (Invitrogen) with primers BT207 and BT208. The Drk1 fragment was then inserted into pDONR207 (Invitrogen) using the Gateway Cloning System and BP Clonase (Invitrogen).

pRS426 TEG1-GST(S93A)-Ypd1. For *in vivo* Ypd1 phosphorylation assays, we initially used the pRS426 TEG1-Ypd1 plasmid (18). To circumvent phosphorylation of the GST tag on Ypd1 (60), we mutated the GST serine 93 to alanine using the QuikChange II site-directed mutagenesis kit (Agilent) with primers SML155 and SML156.

pGEX-KG-Drk1. Drk1 flanked by NdeI and XhoI was cloned into pGEX after Ypd1 was removed using XhoI and XbaI, and an NdeI site was introduced with oligonucleotides BT539 and BT540.

FIG 7 Proposed model of fludioxonil mechanism of action. Based on our data that fludioxonil does not induce Drk1 to dephosphorylate Ypd1 *in vitro*, we propose a new potential mechanism of action. Here, fludioxonil does not directly target Drk1. Rather, it acts upon an upstream target, leading to a change in the intracellular milieu, for example, due to oxidative stress. Drk1 senses this disruption in milieu and converts from kinase to phosphatase activity, enabling it to dephosphorylate Ypd1 and activate the HOG pathway.

pGEX-KG-Drk1R. To make pGEX-KG-Drk1R, we amplified the Drk1 receiver domain (bp 3253 to 3864) from pGEX-KG-Drk1 using Herculase II DNA polymerase (Agilent Technologies, Santa Clara, CA) and primers SML136 and SML138 and ligated it into the pGEX-KG backbone after removal of GST-ScYpd1 with XhoI and XbaI.

pAG423-GAL-Drk1 and pYES-DEST52-Drk1. We used the Gateway system to make pAG423-GAL-Drk1 and pYES-DEST52-Drk1. To do this, we performed an LR reaction with LR Clonase II (Invitrogen), the donor vector (pDONR-Drk1), and the destination vector (pAG423GALccdB [a gift from Susan Lindquist; Addgene plasmid 14149] or pYES-DEST52 [61]).

Drk1 D1140N mutagenesis. To mutate the conserved aspartate to asparagine in full-length Drk1 or Drk1R, we used the QuikChange II site-directed mutagenesis kit with the primers SML151 and SML152.

Sequencing. After cloning, all sequences were analyzed using BigDye Terminator v. 3.1 mix (Applied Biosystems) to check for mutations. After cycling (96°C for 2 min, and then 35 cycles of 96°C for 10 s, 52°C for 15 s, and 60°C for 3 min, followed by 1 min at 72°C), reactions were cleaned up using a CleanSeq magnetic bead sequencing reaction clean-up kit (Agencourt Bioscience). The samples were sequenced at the UW-Madison Biotechnology Center, where they were resuspended off the beads in 50 μ l of double-distilled water (ddH₂O). Ten microliters of each sample was aliquoted into a 96-well PCR plate and mixed with an additional 10 μ l of ddH₂O, and the plate was loaded onto the sequencers according to the manufacturer's instructions. Samples were electrophoresed on an Applied Biosystems 3730xl automated DNA sequencing instrument using 50-cm capillary arrays and POP-7 polymer. Mutations were corrected with QuikChange II (Agilent) and resequenced.

Escherichia coli and S. cerevisiae vector transformation. *E. coli* was transformed by electroporation (62). *S. cerevisiae* was transformed using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method with the addition of 100 mM dithiothreitol (DTT) (63).

Nik1 knockout construction of C. albicans. Nik1 gene deletion was performed using fusion PCR, as in Noble and Johnson (64). K1 *C. albicans* genomic DNA was isolated using a MasterPure yeast DNA purification kit (Epicentre). We used the primer pairs listed in Table 3 to amplify the 5' Nik1 flanking region, the 3' Nik1 flanking region, the NAT1 marker, and the HygB marker, and we used *Ex Taq* (TaKaRa) for both the amplification and fusion PCRs. We replaced the first Nik1 allele with NAT1 and the second allele with HygB. Fusion PCR products were transformed into *C. albicans* K1 with electroporation (65) and

TABLE 1 Strains used in this study

Name	Background	Description/plasmid(s)	Reference or source
<i>B. dermatitidis</i>			
WT 14081	SLH14081	Clinical isolate from the WI State Laboratory of Hygiene	28, 51
Drk1 RNAi	SLH14081		28
WT 26199	ATCC 26199		American Type Culture Collection (ATCC)
<i>S. cerevisiae</i>			
WT BY4741	BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	52, ^a 53
Empty vector	BY4741	WT transformed with pYES-DEST52 empty vector	This work
Drk1	BY4741	WT transformed with pYES-DEST52 Drk1 plasmid	This work
Drk1 D1140N	BY4741	WT transformed with pYES-DEST52 Drk1 D1140N plasmid	This work
GST(S93A)-Ypd1, Drk1	BY4741	WT transformed with pRS426 TEG1-GST S93A-Ypd1 and pAG423 GAL-Drk1 plasmids	This work
GST(S93A)-Ypd1, Drk1 D1140N	BY4741	WT transformed with pRS426 TEG1-GST S93A-Ypd1 and pAG423 GAL-Drk1 D1140N plasmids	This work
WT W303-1A	W303-1A	<i>MATa ade2-1 his3-11,15 leu-2,3, 112 trp1-1 ura3-1 can1-100 GAL mal SUC2</i>	ATCC
ΔHog1	W303-1A	<i>MATa hog1Δ::TRP1</i>	54, ^b 55
Drk1 ΔHog1	W303-1A	ΔHog1 transformed with pYES-DEST52 Drk1 plasmid	This work
<i>E. coli</i>			
GST-Ypd1	DH10β	DH10β transformed with pGEX-KG-Ypd1	This work
GST-Sln1K	DH5α	DH5α transformed with pGEX-KG-Sln1K	56
GST-Sln1R	DH5α	DH5α transformed with pGEX-KG-Sln1R	56
GST-Drk1	BL21(DE3)	BL21(DE3) transformed with pGEX-KG-Drk1	This work
GST-Drk1R	BL21(DE3)	BL21(DE3) transformed with pGEX-KG-Drk1R	This work
GST-Drk1R D1140N	BL21(DE3)	BL21(DE3) transformed with pGEX-KG-Drk1R D1140N	This work
<i>A. fumigatus</i>	Af293		Fungal Genetics Stock
<i>C. albicans</i>			
WT 18804	18804		ATCC
WT K1	K1		D. Andes ^c
Nik1 ^{-/+}	K1	Single Nik1 allele knockout	This work
Nik1 ^{-/-}	K1	Double Nik1 allele knockout	This work
<i>C. gattii</i>	WM276, serotype B	Clinical isolate	Personal communication ^d
<i>C. neoformans</i> var. <i>grubii</i>	H99	<i>MATα</i> , serotype A	ATCC
<i>Rhizopus oryzae</i> 04335-8297		Clinical isolate	UW Hospital and Clinic Clinical Microbiology Laboratory (57)

^aStrain was generously provided by Audrey Gasch.^bStrain was generously provided by Jose Prieto.^cStrain was generously provided by David Andes.^dStrain was generously provided by Dee Carter from the University of Sydney, Australia.

plated on either 200 μg/ml nourseothricin or 600 μg/ml hygromycin B. Double mutants were selected on both antibiotics.

Fludionoxil disc diffusion assay. Disc diffusion assays were performed using a top agar method (57). Briefly, 0.6 ml of 0.25% agarose in medium was inoculated with fungi and poured onto a bed of solidified medium with 2% agar in a 6-well plate. Agar was inoculated with either 6×10^5 conidia/well (*A. fumigatus* and *Rhizopus*), 1.2×10^6 yeast/well (*B. dermatitidis* 14081), or 1×10^4 yeast/well (*C. albicans* 18804, *Cryptococcus gattii*, and *Cryptococcus neoformans*). After the top agar solidified, sterile 6-mm paper discs containing 1 μl of DMSO or 1, 10, or 100 μg of fludionoxil (Sigma) dissolved in 1 μl of DMSO were placed in the center of each well. The plates were incubated at 30°C (*C. gattii*, *C. neoformans*, and *Rhizopus*), 37°C (*A. fumigatus* and *B. dermatitidis*), or room temperature (*C. albicans*) for 24 h (*A. fumigatus* and *Rhizopus*), 48 h (*C. gattii* and *C. neoformans*), 72 h (*B. dermatitidis*), or 96 h (*C. albicans*). Results reported represent the lowest concentration of fludionoxil that inhibited fungal growth (1 μg fludionoxil for *A. fumigatus*, *B. dermatitidis*, and *C. gattii* or 100 μg fludionoxil for *C. albicans*, *C. neoformans*, and *Rhizopus*).

***C. albicans* and *S. cerevisiae* fludionoxil growth assay.** Overnight cultures of *C. albicans* or *S. cerevisiae* were diluted to a final optical density at 600 nm (OD_{600}) of 0.1 and transferred into round-bottomed 96-well plates with 1.25 μg/ml fludionoxil (in 1% DMSO) or 25 μg/ml fludionoxil (in 1% DMSO) for *C. albicans* or *S. cerevisiae*, respectively. DMSO (1%) was used as a solvent control. Cells were incubated for 24 h (*C. albicans*) or 48 h (*S. cerevisiae*). Growth was measured at the OD_{600} in a plate reader (*C. albicans*) or determined visually by accumulation of cells in the bottom of the well (*S. cerevisiae*).

TABLE 2 Plasmids used in this study

Name	Parent/description	Reference or source
pRS426 TEG1-Ypd1	pRS426 TEG1 with Ypd1 insert	18 ^a
pRS426 TEG1- GST(S93A)-Ypd1	pRS426 TEG1-Ypd1 with S93 of the GST tag mutated to alanine	This work
pAG423 GAL-Drk1	pAG423 GAL-CcdB with Drk1 insert	This work
pAG423 GAL-Drk1 D1140N	pAG423 GAL-Drk1 with Drk1 D1140 mutated to asparagine	This work
pYES-DEST52 Empty Vector		Invitrogen Life Technologies
pYES-DEST52 Drk1	pYES-DEST52 with Drk1 insert	This work
pYES-DEST52 Drk1 D1140N	pYES-DEST52 Drk1 with Drk1 D1140 mutated to asparagine	This work
pGEX-KG-Ypd1 (pOU1)	pGEX-KG (69) with Ypd1 inserted with XbaI (5') and XhoI (3')	A. West ^b
pGEX-KG-Sln1K (pHL581)	pGEX-KG with Sln1K (aa 537 to 947) insert	56 ^c
pGEX-KG-Sln1R (pADA689)	pGEX-KG with Sln1R (aa 1068 to 1220) insert	56 ^c
pGEX-KG-Drk1	pGEX-KG with Drk1 insert	This work
pGEX-KG-Drk1R	pGEX-KG with Drk1R (aa 1085-1288) insert	This work
pGEX-KG-Drk1R D1140N	pGEX-KG-Drk1R with Drk1 D1140 mutated to asparagine	This work

^aPlasmid was a generous gift from Haruo Saito.^bPlasmid was a generous gift from Ann West.^cPlasmid was a generous gift from Jan Fassler.

Blastomyces fludioxonil sensitivity. Wild-type and Drk1 RNAi-silenced strain 14081 of *B. dermatitidis* were grown overnight in liquid HMM, backpassed to an OD₆₀₀ of 0.3, and incubated in the presence or absence of 1 μg/ml fludioxonil. Flasks were photographed after 3 days of growth at 37°C, with shaking at 220 rpm.

Blastomyces Hog1 Western blotting. Wild-type and Drk1 RNAi-silenced *B. dermatitidis* strain 14081 were grown overnight in liquid HMM, backpassed to an OD₆₀₀ of 0.3, and again grown overnight before exposure to 1 μg/ml fludioxonil. After incubation, cells were collected by centrifugation and the supernatant was removed. The remaining pellet was flash frozen in an ethanol-dry ice bath. The cell pellet next was broken using a sterile pipette tip, an equal volume of 0.5-mm glass beads was added, and the cells were lysed by bead beating for 1 min, followed by 1 min on ice for 6 min total. The lysate was centrifuged to pellet the beads and insoluble material, and the clarified lysate was transferred to a clean

TABLE 3 Primers used in this study

Name	Sequence (5'→3')	Purpose/target
AJM122	GGTCACTACACCATCGGAAAG	RT-PCR for <i>B. dermatitidis</i> tubulin subunit alpha-2 (forward)
AJM123	CTGGAGGGACGAACAGTTG	RT-PCR for <i>B. dermatitidis</i> tubulin subunit alpha-2 (reverse)
BT207	GGGGACAAGTTTGTACAAAAAAGCAGGCTAAAAATGACTCGGGGTGATGAAAC	Adds <i>attB</i> site and amplifies Drk1 for Gateway cloning (forward)
BT208	GGGGACCACTTTGTACAAGAAAGCTGGGTCTAATCTTTAGTCCACGAAC	Adds <i>attB</i> site and amplifies Drk1 for Gateway cloning (reverse)
BT254	ATTGGGGCTCTTCAATTCTAA	RT-PCR for <i>B. dermatitidis</i> BdGpd1 (forward)
BT255	TTCCTCATATACCCACATCTGC	RT-PCR for <i>B. dermatitidis</i> BdGpd1 (reverse)
BT268	GAGAAGGCGATTCTAGATGTGA	RT-PCR for <i>B. dermatitidis</i> BdXyl2 (forward)
BT269	AATACTCCACACCACGAATGAG	RT-PCR for <i>B. dermatitidis</i> BdXyl2 (reverse)
BT278	GCTGGAATCATAAACGAAATGA	RT-PCR for <i>B. dermatitidis</i> BdArDH (forward)
BT279	CACCAAACCTTCACTCCAAAT	RT-PCR for <i>B. dermatitidis</i> BdArDH (reverse)
BT312	TATCTGCTCACAAATGGCGGC	RT-PCR for <i>B. dermatitidis</i> BdCut1 (forward)
BT313	CGGTCTCATAGTATTCGGCAAGAG	RT-PCR for <i>B. dermatitidis</i> BdCut1 (reverse)
BT539	CTAGACATATGC	Oligonucleotide to create an NdeI site with XbaI and XhoI sticky ends for cloning pGEX-KG-Drk1 (forward)
BT540	TCGAGCATATGT	Oligonucleotide to create an NdeI site with XbaI and XhoI sticky ends for cloning pGEX-KG-Drk1 (reverse)
IRLK065	TACTATACCCCAAATTGATTC	Amplify the 5' Nik1 flanking region (forward)
IRLK066	CACGGCGCGCCTAGCAGCGGGGAGAACTCGAGATAAACAAAC	Amplify the 5' Nik1 flanking region (reverse)
IRLK067	GTACGGCGCGCATCCCTGCAACCATTTGTGTGTTAGATCA	Amplify the 3' Nik1 flanking region (forward)
IRLK068	AAGGTAGCAACTTTTCTCTCAG	Amplify the 3' Nik1 flanking region (reverse)
IRLK069	CCGCTGCTAGGCGCGCCGTGGATATCAAGCTTGCCTCGTCC	Amplify the NAT1 marker (forward)
IRLK070	GCAGGGATGCGGCGCTGACTTCTCTGCGCACTTAACCTC	Amplify the NAT1 marker (reverse)
IRLK105	CCGCTGCTAGGCGCGCCGTGGTATAGTGCTTGCTGTTGAT	Amplify the HygB marker (forward)
IRLK106	GCAGGGATGCGGCGCTGACATTTATGATGGAATGAATGG	Amplify the HygB marker (reverse)
SML136	GCGCGCTCTAGAGACCACACAGATCGTTGA	Amplifies and adds a 5' XbaI site to Drk1 (forward)
SML138	CGCGCGCTCGAGCTAATCTTTAGTCCACGAAC	Amplifies and adds a 3' XhoI site to Drk1 (reverse)
SML151	GATACGACGTATTTTGTGAATGTCCAAATGCCTGTTATG	Mutate aspartate 1140 of Drk1 to asparagine (forward)
SML152	CATAACAGGCATTTGGACATTCATCAAAATGACGTCGTATC	Mutate aspartate 1140 of Drk1 to asparagine (reverse)
SML155	AACCGCTCCTTCAAGCATGCAATCTCTGCACGCTCTTTTG	Mutate serine 93 of GST to alanine (forward)
SML156	CAAAAGAGCGTGACAGATTGCTATGCTTGAAGGAGCGGTT	Mutate serine 93 of GST to alanine (reverse)

tube. Lysate was run on an SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with Tris-buffered saline-Tween 20 (TBST) plus 5% nonfat dry milk and washed, and phospho-Hog1 was detected using a 1:1,000 dilution of phospho-p38 primary antibody (9215; Cell Signaling Technology, Danvers, MA). Following incubation with an anti-rabbit alkaline phosphatase secondary antibody, the membrane was developed with nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) for alkaline phosphatase detection.

B. dermatitidis RT-PCR. RNA isolation and reverse transcription-PCR (RT-PCR) were performed as described in Marty et al. (66). Briefly, 15-ml cultures of wild-type or Drk1 RNAi *B. dermatitidis* yeast were grown overnight with a starting OD₆₀₀ of 0.8 in HMM, followed by a 90-min incubation with 1 µg/ml of fludioxonil at 37°C. Yeast were collected, washed with phosphate-buffered saline (PBS), and flash frozen in liquid nitrogen. RNA was isolated by grinding frozen cells with a mortar and pestle and dissolving in TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH), followed by separation with 1-bromo-3-chloropropane (Molecular Research Center Inc., Cincinnati, OH). RNA was precipitated using a 1:1 solution of isopropanol and high-salt solution (Molecular Research Center Inc., Cincinnati, OH) and washed with 75% ethanol. Residual DNA was removed by adding 2 U of Turbo DNase (Applied Biosystems/Ambion, Austin, TX) to 10 µg of RNA in a 50-µl reaction mixture and further purified using an RNeasy kit (Qiagen), with an extra RPE buffer wash to remove salts. RNA integrity was assessed using NanoDrop spectrophotometry (NanoDrop Products, Wilmington, DE) and 0.8% agarose gel electrophoresis.

cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad, Inc., Hercules, CA). Real-time PCRs consisted of 1 × IQ SYBR green supermix (Bio-Rad, Inc., Hercules, CA), 0.5 µM each primer, and 5 µl of 1:10 diluted cDNA in a 25-µl reaction mixture. All reactions were performed in triplicate. Real-time PCR was performed using a Bio-Rad iCycler MyiQ. Cycling conditions were 1 cycle at 95°C for 1.5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Melting curve analysis was performed after the completion of the PCR. Gene expression was normalized relative to the expression of alpha-tubulin, and fold change is relative to gene expression at time zero (67). Fold change equals $2^{-\Delta\Delta C_T}$, where C_T is threshold cycle and $\Delta\Delta C_T$ is $(C_{T, \text{target}} - C_{T, \text{tubulin}})_{90 \text{ min}} - (C_{T, \text{target}} - C_{T, \text{tubulin}})_{0 \text{ min}}$. Primers for RT-PCR analysis are shown in Table 3.

S. cerevisiae Hog1 Western blotting. Empty vector and Drk1 *S. cerevisiae* strains, grown in SC-Ura plus raffinose to an OD₆₀₀ of 0.5, were transferred to SC-Ura plus galactose medium for 4 h to allow for Drk1 expression. The cultures were then divided into 10-ml aliquots and exposed to either 0.4 M NaCl or 25 µg/ml fludioxonil for 5 min. Cells were collected by centrifugation, and cell pellets were frozen in liquid nitrogen. The cell pellets were resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 15 mM EDTA, 15 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 2 mM DTT, 0.1% Triton X-100, cOmplete EDTA-free protease inhibitor cocktail tablets [Roche]) plus 10 µg/ml pancreatic RNase A (Sigma) and lysed by vortexing with 0.5-mm acid-washed glass beads for 10 min, alternating 30 s of vortexing and 30 s on ice. The lysate was centrifuged for 10 min at 10,000 rpm and run on an SDS-PAGE gel before transferring to a PVDF membrane using the TransBlot turbo semidry system (Bio-Rad). After blocking in 5% nonfat dry milk in TBST, the membranes were incubated with phospho-Hog1 (1:1,000; no. 9215; Cell Signaling) or total Hog1 primary antibody (1:200; yC-20; Santa Cruz Biotechnology), followed by secondary antibody (1:2,000 goat anti-rabbit horseradish peroxidase [HRP; 111-035-045; Jackson ImmunoResearch Laboratories] or 1:10,000 rabbit anti-goat HRP [sc-2768; Santa Cruz Biotechnology]). Membranes were developed with Clarity Western ECL substrate (Bio-Rad) and imaged with VersaDoc Imaging Systems (Bio-Rad).

In vivo Ypd1 phosphorylation. To measure Ypd1 phosphorylation *in vivo* in *S. cerevisiae*, we adapted the protocol from Posas et al. (18). Briefly, an overnight culture of *S. cerevisiae* expressing GST(S93A)-Ypd1 and pAG423 Drk1 or pAG423 Drk1 D1140N in SC-Ura-His plus glucose (uninduced) or galactose (induced) medium was backpassed to an OD₆₀₀ of 0.3. Once an OD₆₀₀ of ~1.0 was reached, cultures were centrifuged and resuspended to an OD₆₀₀ of ~10 in phosphate-depleted SC-Ura-His plus glucose or galactose (68) and incubated for 5 min with shaking. An aliquot of 0.75 mCi/ml of [³²P]orthophosphate (~9,000 Ci/mmol, 10 mCi/ml; NEX053H; PerkinElmer) was added, and the cultures were incubated an additional 5 min. The cells were then exposed to 0.4 M NaCl for 15 min, followed by a 5-min osmotic shock by adding 9 volumes of water. An aliquot was removed at this point for the starting sample and kept on ice. The remaining sample was exposed to either 0.4 M NaCl for 1 min or 25 µg/ml fludioxonil for 15 min. The cells were harvested and lysed as described above for *S. cerevisiae* Hog1 phosphorylation assays. Following centrifugation of the lysate, glutathione Sepharose 4B resin (GE Healthcare Life Sciences) was added to the clarified lysate and incubated for 25 min at 4°C to purify Ypd1. The resin was then washed once with buffer A, followed by four washes with buffer A plus 150 mM NaCl, and resuspended in an equal volume of 2× SDS loading buffer. After running the samples on an SDS-PAGE gel, the gel was exposed to a phosphorimaging screen overnight at room temperature and subsequently stained with Coomassie R-250 for total protein analysis. The phosphorimaging screen was read with a Typhoon FLA9000 (GE Healthcare Life Sciences), and we calculated densitometry using ImageQuant TL (GE Healthcare Life Sciences).

Protein expression and purification. Overnight cultures of *E. coli* expressing Sln1, Ypd1, or Drk1 (Table 1) were grown, with shaking, at 37°C, diluted to an OD₆₀₀ of 0.1 in a final volume of 200 ml, and grown at 37°C to an OD₆₀₀ of ~0.9. To induce protein expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 0.2 mM and the cultures were incubated for 24 h, with shaking, at 15°C. After pelleting the cells by centrifugation, cells were resuspended in 4 to 5 ml of lysis buffer (50 mM Tris-Cl, pH 8, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% β-mercaptoethanol, Roche EDTA-free protease inhibitor tablet) per gram of cell pellet wet weight and lysed by sonication (Sln1K, Sln1R, Ypd1, and Drk1R) or French press (Drk1). Crude lysates were centrifuged at 27,200 × g for 30 min. The supernatant was removed and incubated for 2 h with 1 ml glutathione Sepharose 4B resin at 4°C with gentle rocking

followed by column purification. Resin was washed with 40 ml of lysis buffer and 40 ml of storage buffer (50 mM Tris-Cl, pH 8.0, 100 mM KCl, 2 mM DTT, 1 mM EDTA, 10% glycerol) and then suspended to a 50% slurry in storage buffer and stored at -20°C . The GST tags were removed from Sln1K and Sln1R using a thrombin cleavage capture kit (EMD Millipore). Roughly 4 mg of each protein was incubated with ~ 6 U of thrombin for 2 (Sln1K) or 4 (Sln1R) h.

Drk1 kinase assay. The *in vitro* kinase reaction was adapted from Li et al (56). Resin-bound GST-Ypd1 (1 μg) and GST-Drk1 (1 μg) were incubated in reaction buffer (50 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl_2 , 2 mM DTT) with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (30 μM total ATP, 30 Ci/mmol total, 18 μCi per reaction, using BLU502Z; PerkinElmer) at room temperature for 45 min. $6\times$ SDS-sample buffer was added to stop the reaction, and samples were run on SDS-PAGE, followed by exposure of the gel to a phosphorimaging screen. Phosphorimaging was performed as described above.

***In vitro* Ypd1 phosphorylation assays.** Resin-bound GST-Ypd1 (5 μg per sample) was phosphorylated by 1 μg Sln1K and 1 μg Sln1R in reaction buffer for 30 min at room temperature following the addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (30 μM total ATP, 30 Ci/mmol total, 18 μCi per reaction, using BLU502Z; PerkinElmer). Sln1K, Sln1R, and unincorporated ATP were washed away from GST-Ypd1, and the remaining GST-Ypd1 was resuspended in reaction buffer with 1 mM ATP and added to either GST-Drk1 (50 μg), GST-Drk1R (25 μg), or GST-Drk1R D1140N (25 μg), as indicated. For fludioxonil experiments, either DMSO or fludioxonil (in DMSO) was added to a final concentration of 2% and up to 2 mg/ml, respectively. Samples were collected at the indicated time points and $6\times$ SDS sample buffer was added to stop the reaction, but samples were not boiled so as to prevent hydrolysis of the phosphohistidine. Samples were run on SDS-PAGE, and the gel was exposed to a phosphorimaging screen. Phosphorimaging was performed as described above.

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