MAPK Hog1 closes the *S. cerevisiae* glycerol channel Fps1 by phosphorylating and displacing its positive regulators

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The aquaglyceroprin Fps1 is responsible for glycerol transport in yeast in response to changes in extracellular osmolarity. Control of Fps1 channel activity in response to hyperosmotic shock involves a redundant pair of regulators, Rgc1 (regulator of the glycerol channel 1) and Rgc2, and the MAPK Hog1 (high-osmolarity glycerol response 1). However, the mechanism by which these factors influence channel activity is unknown. We show that Rgc2 maintains Fps1 in the open channel state in the absence of osmotic stress by binding to its C-terminal cytoplasmic domain. This interaction involves a tripartite pleckstrin homology (PH) domain within Rgc2 and a partial PH domain within Fps1. Activation of Hog1 in response to hyperosmotic shock induces the rapid eviction of Rgc2 from Fps1 and consequent channel closure. Hog1 was recruited to the N-terminal cytoplasmic domain of Fps1, which it uses as a platform from which to multiply phosphorylate Rgc2. Thus, these results reveal the mechanism by which Hog1 regulates Fps1 in response to hyperosmotic shock.

[*Keywords*: Hog1; Fps1; Rgc1; Rgc2; osmotic shock; phosphoproteomics]

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Under conditions of high osmolarity stress, many fungal species, including *Saccharomyces cerevisiae*, maintain osmotic equilibrium by producing and retaining high concentrations of glycerol as a compatible solute (Nevoigt and Stahl 1997). Intracellular glycerol concentration is regulated in *S. cerevisiae* in part by the Fps1 plasma membrane glycerol channel (Luyten et al. 1995; Sutherland et al. 1997; Tamás et al. 1999). Increased external osmolarity induces Fps1 closure, whereas decreased osmolarity causes channel opening, both within seconds of the change in external osmolarity (Tamás et al. 1999). This channel, which functions as a homotetramer (Beese-Sims et al. 2011), is required for survival of a hypo-osmotic shock, when yeast cells must export glycerol rapidly to prevent bursting (Luyten et al. 1995; Tamás et al. 1999). Fps1 is also required for controlling turgor pressure during fusion of mating yeast cells (Philips and Herskowitz 1997).

Fps1 is a member of the major intrinsic protein (MIP) family of channel proteins. The MIP family is subdivided into members that are selectively permeable to water (aquaporins) and those permeated by glycerol and to a lesser extent by water, called aquaglyceroporins or glycerol facilitators (Borgnia and Agre 2001; Agre et al. 2002). Relative to nonfungal aquaglyceroporins, Fps1 possesses N-terminal and C-terminal cytoplasmic extensions that are important for its regulation (Tamás et al. 2003; Hedfalk et al. 2004). The pathway responsible for regulation of Fps1 in response to changes in osmolarity has not been fully delineated but involves the MAPK Hog1 (high-osmolarity glycerol response 1) (Tamás et al. 1999; Hohmann 2009; Ahmadpour et al. 2013), a homolog of the mammalian p38 MAPK, which binds to the N-terminal cytoplasmic domain of Fps1 (Mollapour and Piper 2007). Hog1 is activated in response to hyperosmotic stress to mediate

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both the biosynthesis of glycerol and its retention within the cell (Hohmann 2009). Although Hog1 plays a role in glycerol retention by control of Fps1, the mechanism by which it influences Fps1 activity is unknown. Hog1 function has been mainly associated with the regulation of transcriptional events (Reiser et al. 1999; Rep et al. 1999; de Nadal and Posas 2010; de Nadal et al. 2011; Saito and Posas 2012), although a recent quantitative mass spectrometry (MS) analysis has revealed several new candidate substrates of Hog1 (Reiter et al. 2012).

Fps1 activity is also controlled by a pair of redundant positive regulators, named Rgc1 (regulator of the glycerol channel 1) and Rgc2 (*YPR115W* and *YGR097W*, respectively) (Beese et al. 2009). Additional genetic analyses suggested that Hog1 is a negative regulator of Rgc1 and Rgc2, and electrophoretic bandshift assays revealed that Rgc2 is phosphorylated in response to hyperosmotic shock in a manner partially dependent on Hog1, suggesting that Hog1 may regulate Fps1 activity indirectly through Rgc1 and Rgc2 (Beese et al. 2009). However, the mechanism by which Rgc1 and Rgc2 control Fps1 activity and its relationship to Hog1 activity remain unclear.

Loss of either *FPS1* or *RGC1* and *RGC2* function results in excess turgor pressure and consequent cell wall stress (Beese et al. 2009). Additional cell wall stress imposed on these mutants by, for example, growth at elevated temperature results in cell lysis. Although the fungal kingdom is replete with Rgc orthologs, they are not represented in metazoans, suggesting that the Rgc–Fps pathway may be an attractive target for antifungal drug development. Indeed, loss of the Fps glycerol channels in the fungal pathogen *Candida glabrata* sensitizes cells to antifungal agents that target the cell wall (Beese-Sims et al. 2012).

In this study, we explore the mechanisms by which Hog1 and Rgc1/2 control Fps1 channel activity in response to hyperosmotic shock. We demonstrate that Rgc2 maintains Fps1 in an open channel state through an association between the pleckstrin homology (PH) domain of Rgc2 and a partial PH domain within the C-terminal domain of Fps1. We identify several Hog1 phosphorylation sites on Rgc2 by mass spectrometric analysis and demonstrate that phosphorylation of these sites is critical for displacement of Rgc2 from the Fps1 C-terminal domain and consequent closure of the channel. Additionally, we show that active Hog1 induces Fps1 closure by binding to a docking site within the Fps1 N-terminal domain, which serves as a platform from which Hog1 phosphorylates Rgc2.

Results

Hog1 regulates association of Rgc2 with the glycerol channel Fps1

Our earlier work suggested that Hog1 may regulate Fps1 activity indirectly through a pair of cytoplasmic proteins, Rgc1 and Rgc2 (Beese et al. 2009). Therefore, to test the possibility that Rgc2 forms a complex with Fps1, we tested by coimmunoprecipitation (co-IP) for association of Rgc2-HA with Fps1-Myc. Figure 1A shows that Rgc2 associates with Fps1 in vivo under nonstress conditions. Interestingly, this association was greatly diminished in response to hyperosmotic shock with 1.8 M sorbitol (Fig. 1B). The majority of Rgc2 dissociates from Fps1 within 10 sec of hyperosmotic shock and remains dissociated for at least 20 min. To determine whether the regulated association of Rgc2 with Fps1 is influenced by Hog1, we examined this association in a *hog1*Δ strain. In the absence of Hog1, Rgc2 was stabilized on Fps1 in response to hyperosmotic shock (Fig. 1B), suggesting that Hog1 phosphorylation of Rgc2 may induce its release from Fps1. These data, combined with our previous results (Beese et al. 2009), suggest that Rgc2 (and likely its redundant paralog, Rgc1) maintains Fps1 in an open channel conformation through its physical interaction with Fps1. They further suggest that Hog1 induces channel closure by the eviction of Rgc2 (and presumably Rgc1) from Fps1.

To detect an interaction between Hog1 and Rgc2 (and Rgc1), we employed a novel assay designed to detect short-lived protein–protein interactions in vivo (Zuzuarregui et al. 2012). The M-track assay is based on fusion of a histone lysine methyltransferase to one protein and fusion of its substrate (the N terminus of histone H3) to another. Using this assay, we were able to detect a hyperosmotic shock-induced interaction between Hog1 and Rgc2 and between Hog1 and Rgc1 (Supplemental Fig. S1).

Hog1 multiply phosphorylates Rgc2

Rgc2 becomes highly phosphorylated in response to hyperosmotic shock (Beese et al. 2009). These phosphorylations are partly dependent on the MAPK Hog1, suggesting a direct regulatory role of the kinase in Rgc2 function, although Hog1-independent phosphorylation of Rgc2 has also been observed during logarithmic growth as well as in response to hypo- and hyperosmotic stress (Beese et al. 2009; Reiter et al. 2012). Currently, 19 phosphorylation sites of Rgc2 are documented in the PhosphoPep (part of the *Saccharomyces* Genome Database) and PhosphoGRID databases (King et al. 2006; Stark et al. 2010), two of which (phosphoserines 344 and 1021) lie within S/T-P MAPK consensus motifs. However, Hog1-dependent regulation of specific phosphorylation sites of Rgc2 has not been described to date.

To unravel the phosphorylation patterns of Rgc2, we conducted MS analysis based on tandem affinity purification, as described (Reiter et al. 2012). In total, our analysis of tryptic and chymotryptic digests covered 65.5% of the Rgc2 protein sequence. Within the covered regions, we were able to identify 30 phosphorylation sites with high confidence, seven of which have been assigned to S/T-P motifs (Fig. 1C,D; Supplemental Table S4). The seven phosphorylated S/T-P sites are phosphoserines 75, 344, 827, 948, 1021, and 1035 and phosphothreonine 808.

We next used quantitative MS to identify sites phosphorylated directly by Hog1. We made use of stable isotope labeling by amino acids in cell culture (SILAC) technology (Ong et al. 2002) to pinpoint phosphorylation sites of Rgc2 susceptible to a novel Hog1 inhibitor (Diner et al. 2011). We also performed SILAC experiments to identify hyperosmotic stress-induced changes in the Rgc2 phosphoryla-

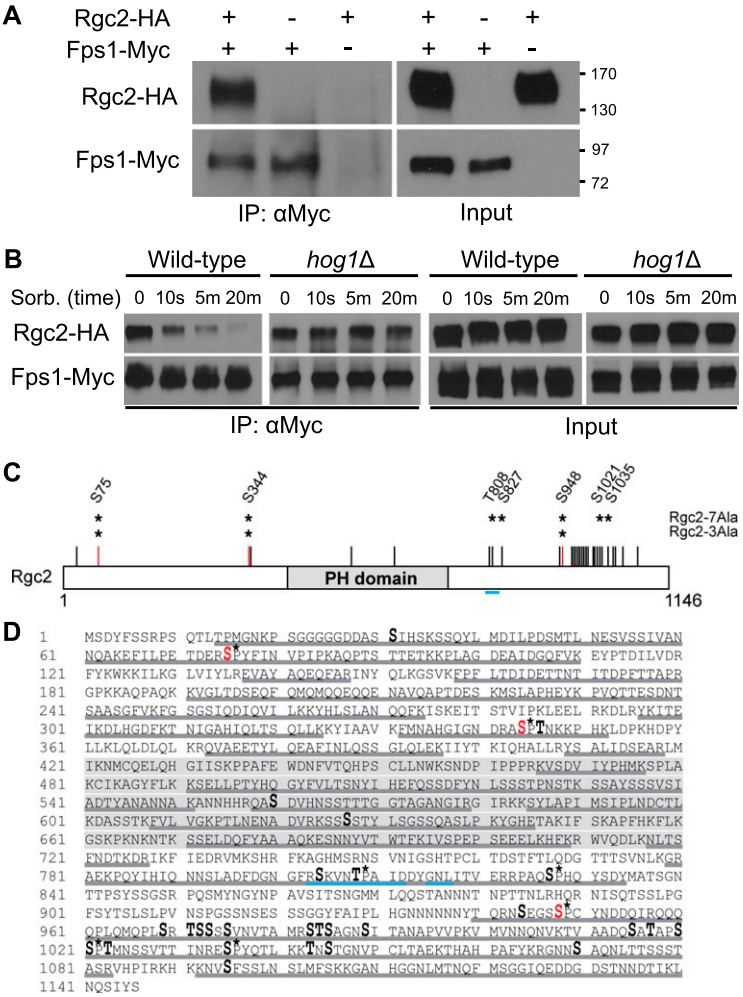

Figure 1. The association of Rgc2 with Fps1 is regulated by Hog1. (*A*) Co-IP of Rgc2 with Fps1. Fps1-Myc was tested for co-IP of Rgc2-HA from extracts of wild-type cells (DL3187) coexpressing differentially tagged Rgc2 and Fps1. Immunoprecipitates were separated by SDS-PAGE and subjected to immunoblot analysis. Controls were from cells that did not express one of the tagged proteins. Molecular mass markers (in kilodaltons) are shown at the *right*. (*B*) Hog1 is required to diminish the Rgc2–Fps1 interaction in response to hyperosmotic shock. Sorbitol was added to cultures of a wild-type (DL3187) or *hog1*Δ (DL3158) strain coexpressing Rgc2-HA and Fps1-Myc to a final concentration of 1.8 M and incubated for the indicated times. (*C*,*D*) Constitutive and stress-regulated phosphorylation sites on Rgc2. Rgc2-HTBeaq (Reiter et al. 2012) was affinity-purified and subjected to mass spectrometric phosphorylation mapping. (*C*) Schematic of Rgc2 showing the clustering of phosphorylation sites identified by MS. Confirmed Hog1 phosphorylation sites are indicated by red bars. Phosphorylated S/T-P motifs mutated in the *RGC2-3A* and *RGC2-7A* alleles are indicated by asterisks. The central PH domain is shown in gray. The blue underline indicates a region that is highly conserved with Rgc1 and contains the only phosphorylated S/T-P site detected that is conserved between the two proteins. (*D*) Rgc2 phosphorylation map: Covered sequences are underlined in dark gray. Phosphorylation sites are shown in bold. Other markings are as in *C*. The details of mass spectrometric analysis of phosphopeptide sites are provided in the Supplemental Material.

tion pattern. Phosphorylation of S/T-P sites that were both blocked by Hog1 inhibitor treatment and induced in response to hyperosmotic stress (when also covered in the osmotically stressed sample) were concluded to be Hog1 target sites (Supplemental Tables S3, S6).

We were able to assign three of the seven phosphorylated S/T-P sites unambiguously as Hog1 targets using our quantitative MS approach. Our analysis identified an inhibitor-induced down-regulation at two phosphorylated S/T-P sites: phosphoserines 75 and 344. Furthermore, we observed an up-regulation of the unphosphorylated peptide covering Ser948, indicating that this peptide becomes dephosphorylated in response to down-regulation of Hog1 activity (Supplemental Table S3). Moreover, phosphoserines 344 and 948 (the latter identified as double-phosphorylated peptide 944/948) were up-regulated upon exposure to hyperosmotic stress.

Results obtained for the four remaining S/T-P motifs (phosphoserines 827, 1021, and 1035 and phosphothreonine 808) did not allow confident assignment as Hog1 target sites. For instance, phosphothreonine 808 was identified as being up-regulated by hyperosmotic stress; however, neither the phosphopeptide nor the unphosphorylated variant was covered in the inhibitor-treated samples. On the other hand, both the phosphorylated and unphosphorylated forms of the peptide covering Ser1021 were detected in the inhibitor-treated samples but not in the stress-treated samples. Although the level of unphosphorylated peptide covering Ser1021 was found to be increased upon inhibitor treatment, the presence of multiply phosphorylated variants of the peptide prevented a confident conclusion regarding Hog1 dependency of this site. Additionally, phosphoserine 827 was identified only in the nonquantitative experiments, precluding its confident assignment as a Hog1 target site.

Taken together, our analysis confirmed phosphorylations at seven S/T-P motifs of Rgc2. We also provide evidence that at least three of these (Ser75, Ser344, and Ser948) are phosphorylated directly by Hog1. A similar phosphoproteomic analysis was carried out for Rgc1 that resulted in the assignment of two confirmed Hog1 sites (Supplemental Fig. S1; Supplemental Tables S3, S4, S6). Because *RGC1* and *RGC2* are functionally redundant with regard to Fps1 function (Beese et al. 2009), we chose to focus our attention on Rgc2. We therefore constructed two types of Rgc2 mutants for further genetic analyses: one

with all seven discovered phosphorylated S/T-P sites mutated to alanine (*RGC2-7A*) and one with the corresponding mutations only affecting the three established Hog1 sites (*RGC2-3A*).

Hog1 phosphorylation of Rgc2 induces its dissociation from Fps1

As demonstrated above, hyperosmotic shock induces rapid dissociation of Rgc2 from Fps1. To assess the role of Rgc2 phosphorylation by Hog1 in the dynamics of the Rgc2–Fps1 interaction, we examined the Rgc2-3A and Rgc2-7A mutant forms for their ability to dissociate from Fps1 in response to hyperosmotic shock. The Rgc2-3A protein was partially stabilized on Fps1 (Fig. 2A). However, the Rgc2-7A protein remained largely associated with Fps1 after hyperosmotic shock (Fig. 2A), supporting the conclusion that the role of Hog1 in this setting is to induce the dissociation of Rgc2 from Fps1 by phosphorylation of the former.

The above results suggest that the Rgc2-3A and Rgc2-7A proteins may render Fps1 in a constitutively open state. We tested this possibility in two ways. First, we examined sensitivity to the toxic metalloid arsenite, which enters *S. cerevisae* cells through the Fps1 channel (Wysocki et al. 2001; Thorsen et al. 2006). Loss-of-function mutants in *FPS1* or *RGC1/2* are therefore resistant to the toxicity of arsenite (Wysocki et al. 2001; Beese et al. 2009). Moreover, treatment with arsenite or the related metalloid antimonite activates Hog1, resulting in the closure of Fps1 and consequent arsenite tolerance (Thorsen et al. 2006). The *RGC2-3A* and *RGC2-7A* mutations caused arsenite sensitivity relative to wild-type *RGC2* (Fig. 2B), supporting the conclusion that stabilization of Rgc2 on Fps1 maintains the channel in an open conformation.

We also tested Fps1 channel activity by measuring the levels of intracellular glycerol. However, we detected only modestly reduced intracellular glycerol levels in the *RGC2-3A* and *RGC2-7A* mutants under both basal and hyperosmotic shock conditions (Fig. 2C). Therefore, we considered the possibility that the cells might be compensating for increased glycerol efflux through elevated glycerol production. *S. cerevisiae* regulates glycerol production through the transcriptional induction of *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, the first committed and rate-limiting step in glycerol biosynthesis (Albertyn et al. 1994; Remize et al. 2001). We found that the basal expression of a *GPD1-lacZ* reporter was elevated in the *RGC2-3A* and *RGC2-7A* mutants (Fig. 2D). The finding of modestly reduced levels of intracellular glycerol despite a presumptive increase in the rate of glycerol production supports the conclusion of elevated glycerol efflux in these mutants.

The PH domain of Rgc2 is required for its association with Fps1

Rgc1 and Rgc2 possess a centrally located but triply split PH domain (Supplemental Fig. S2; Fadri et al. 2005). Split PH domains have been implicated in mediating protein–protein interactions through complementary partial PH

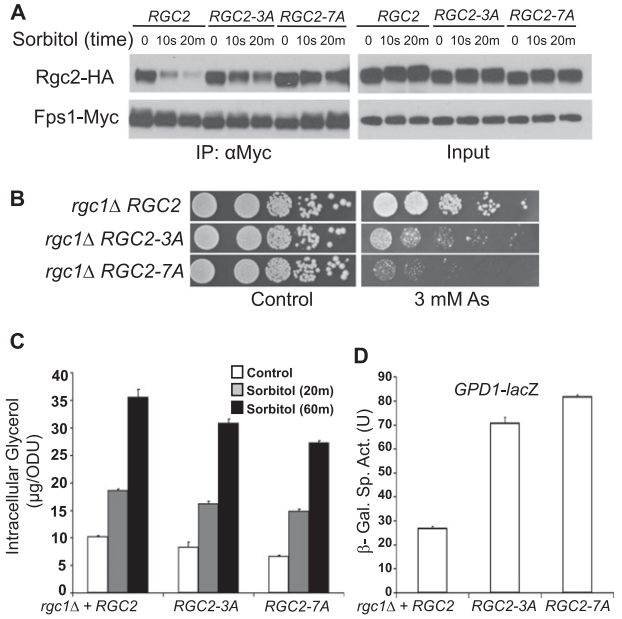

Figure 2. Mutants in Rgc2 at Hog1 phosphorylation sites stabilize Rgc2 on Fps1 and cause open channel phenotypes. (*A*) Co-IP of Hog1 phosphorylation site mutants of Rgc2 with Fps1. Fps1-Myc was coexpressed in wild-type cells (DL3187) with mutant forms of Rgc2-HA and treated as in Figure 1B. Three confirmed Hog1 sites were mutated to Ala residues in the *RGC2-3A* allele. In addition to these mutations, the *RGC2-7A* allele possesses four more Ala mutations at suspected Hog1 phosphorylation sites. (*B*) Arsenite hypersensitivity of *RGC2-3A* and *RGC2-7A* mutations. Mutant *RGC2* alleles were integrated into the yeast genome at the *RGC2* locus of a strain bearing an *rgc1*Δ mutation. Equivalent numbers of cells in 10-fold serial dilutions of each strain were spotted onto YEPD plates with or without 3 mM arsenite and incubated for 3 d at 30°C. The yeast strains used were *rgc1*Δ *RGC2* (DL4070), *rgc1*Δ *RGC2-3A* (DL4066), and *rgc1*Δ *RGC2-7A* (DL4062). (*C*) Diminished glycerol accumulation in Hog1 phosphorylation site mutants of *RGC2*. The strains from *B* were used for measurements of intracellular glycerol content in response to hyperosmotic shock with sorbitol at 1.8 M for the indicated times. (*D*) Elevated levels of *GPD1* expression in Hog1 phosphorylation site mutants of *RGC2*. The same strains from *B* were transformed with a *GPD1-lacZ* reporter plasmid to measure differences in the basal level of *GPD1* expression. For *C* and *D*, values are the mean and standard deviation from three independent cultures.

domains in other proteins (van Rossum et al. 2005; Yan et al. 2005). To determine whether the PH domain or another region of Rgc2 is important for Fps1 binding, we created a set of in-frame deletions within the *RGC2* gene as well as truncations of its N-terminal or C-terminal domains (Fig. 3A). We found that deletion of any of the three portions of the PH domain or the entire PH domain (*rgc2-PH123*Δ) severely impaired Rgc2 binding to Fps1, whereas truncation of either the Rgc2 N-terminal or C-terminal domains did not affect its association with Fps1 (Fig. 3B). To address this issue in greater detail, we mutated four residues within the Rgc2 PH domain to Ala that are conserved among PH domains (residues G486, L505, I510, and W711) (Supplemental Fig. S2). Although none of the

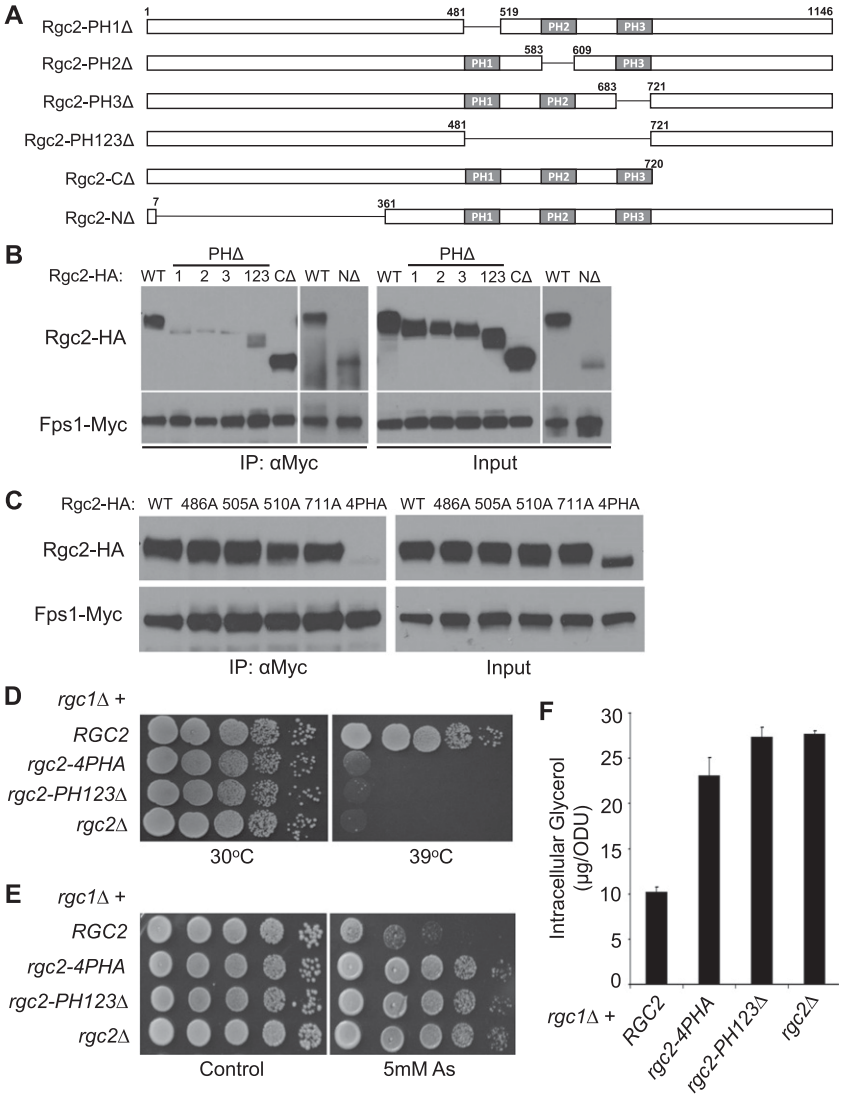

Figure 3. The PH domain of Rgc2 is important for its interaction with Fps1. (*A*) Schematic of Rgc2 showing various deletions within its tripartite PH domain and terminal truncations. (*B*) Co-IP of PH domain deletion mutants of Rgc2 with Fps1. Fps1-Myc was coexpressed in a wild-type strain (DL3187) with mutant forms of Rgc2-HA. (*C*) Co-IP of PH domain point mutants of Rgc2 with Fps1. Fps1-Myc was coexpressed in a wild-type strain (DL3187) with mutant forms of Rgc2-HA. (*D*) Temperature-dependent cell lysis of the *rgc2-4PHA* and *rgc2-PH123*Δ mutants. Equivalent numbers of cells in 10-fold serial dilutions of each strain were spotted onto YEPD plates incubated for 3 d at the indicated temperature. Cell lysis was confirmed by the microscopic appearance of nonrefractile "ghosts." Mutant *RGC2* alleles were integrated into the yeast genome at the *RGC2* locus of a strain bearing an *rgc1*Δ mutation. The yeast strains used were *rgc1*Δ *RGC2* (DL4070), *rgc1*Δ *rgc2-4PHA* (DL4120), *rgc1*Δ *rgc2-PH123*Δ (DL4136), and *rgc1*Δ *rgc2*Δ (DL4046). (*E*) Arsenite resistance of the *rgc2-4PHA* and *rgc2-PH123*Δ mutants. The same yeast strains were spotted onto YEPD plates with or without 5 mM arsenite and incubated for 3 d at 30°C. (*F*) Retention of excess glycerol in the *rgc2-4PHA* and *rgc2-PH123*Δ mutants. The same strains were used for measurements of basal intracellular glycerol content. Values are the mean and standard deviation from three independent cultures.

individual mutations diminished the Rgc2–Fps1 association, the combination of all four (*rgc2-4PHA*) blocked this interaction (Fig. 3C), supporting the conclusion that the Rgc2 PH domain is important for its association with Fps1.

Loss of the ability of Rgc2 to bind Fps1 is predicted to result in a closed channel phenotype in the absence of Rgc1. Consistent with this prediction, neither the *rgc2-PH123*Δ allele, which encodes a form of Rgc2 that is lacking the entire PH domain, nor the *rgc2-4PHA* point mutant form were able to complement the temperature-dependent cell lysis defect of an *rgc1*Δ *rgc2*Δ mutant (Fig. 3D), suggesting that these mutant forms do not function to open Fps1. This conclusion was supported by the additional finding that both of these mutants displayed a level of resistance to arsenite similar to that of the *rgc1*Δ *rgc2*Δ mutant (Fig. 3E). Finally, a defect in Fps1 channel activity was confirmed by the observation that these mutant forms of Rgc2 caused an increase in the basal level of glycerol retention similar to that observed for the *rgc1*Δ *rgc2*Δ mutant (Fig. 3F).

Rgc2 binds to the C-terminal cytoplasmic domain of Fps1

To characterize further the nature of the Rgc2–Fps1 interaction, we turned our attention to the cytoplasmic domains of Fps1. As noted earlier, Fps1 possesses cytoplasmic domains at both its N terminus and C terminus that are thought to be important for its regulation (Tamás et al. 2003; Hedfalk et al. 2004). Deletions that remove either of these extensions result in constitutively open forms of Fps1. Therefore, we asked by co-IP whether either an N-terminal (Fps1-Δ1) (Tamás et al. 2003) or C-terminal (Fps1-C1) (Hedfalk et al. 2004) Fps1 deletion mutant (Fig. 4A) was defective in its association with Rgc2. The C-terminally truncated form of Fps1 failed to associate with Rgc2, whereas the N-terminally truncated form retained its association (Fig. 4B), suggesting that Rgc2 interacts with the Fps1 C-terminal domain.

To map the Rgc2-binding site within Fps1, we constructed a set of nested deletions within the Fps1 C

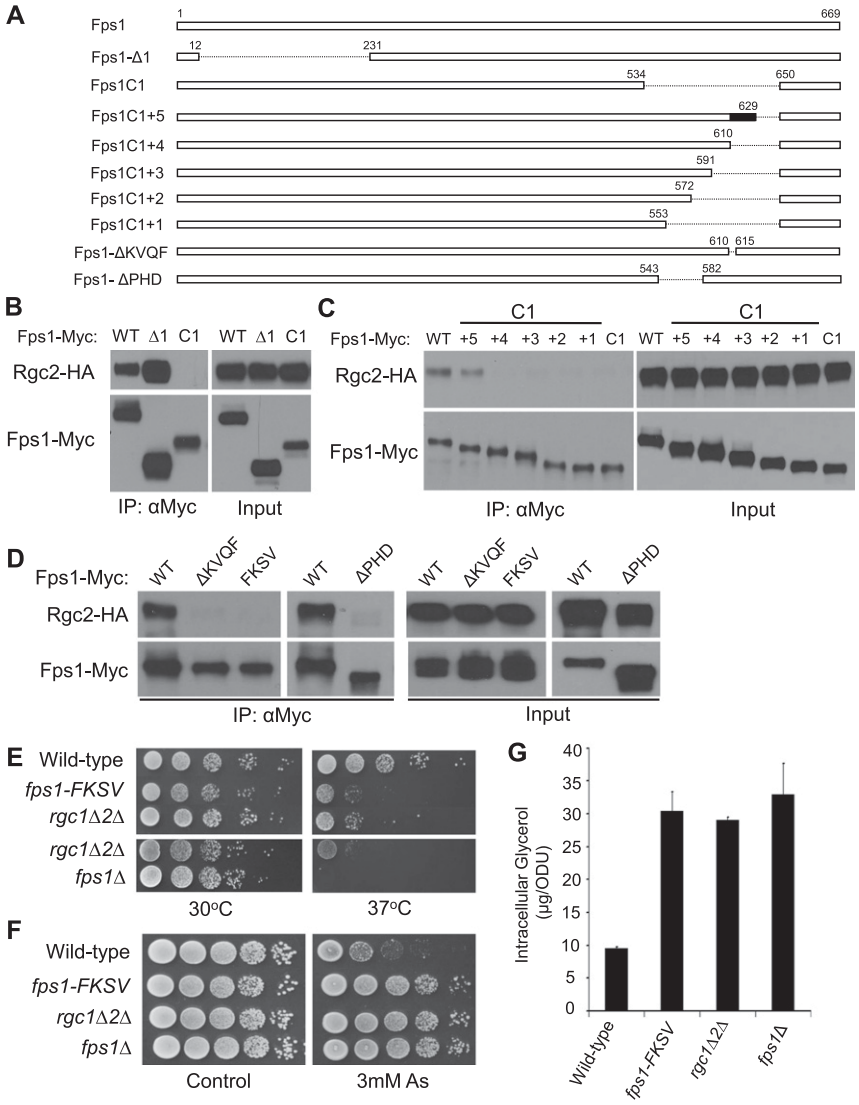

Figure 4. Rgc2 binds to a site within the C-terminal cytoplasmic domain of Fps1. (*A*) Schematic of Fps1 showing various internal deletions tested for association with Rgc2. The region between residues 611 and 629, indicated in black, was identified as important for Rgc2-binding. (*B*) Rgc2 fails to bind to a C-terminal deletion form of Fps1. Co-IP of Rgc2 with N-terminal and C-terminal deletions of Fps1 coexpressed in an *fps1*Δ strain (DL3226). (*C*) Rgc2 binding to mutant forms of Fps1 with nested deletions in the C-terminal domain. (*D*) Rgc2 binding to C-terminal mutant forms of Fps1 coexpressed in an *fps1*Δ strain (DL3226). (*E*) Temperature-dependent cell lysis of the *fps1-FKSV* mutant. Equivalent numbers of cells in 10-fold serial dilutions of each strain were spotted onto YEPD plates incubated for 3 d at the indicated temperature. The yeast strains used were *fps1*Δ (DL3226) expressing *FPS1* (wild type), *fps1-FKSV*, or centromeric vector only (*fps1*Δ) and *rgc1*Δ *rgc2*Δ (DL3207) transformed with vector only. (*F*) Arsenite resistance of the *fps1-FKSV* mutant. The same yeast strains were spotted onto YEPD plates with or without 3 mM arsenite and incubated for 3 d at 30°C. (*G*) Retention of excess glycerol in the *fps1-FKSV* mutant. The same yeast strains were used for measurements of basal intracellular glycerol content. Values are the mean and standard deviation from three independent transformants.

terminus between residues 534 and 650, the limits of the Fps1-C1 deletion (Fig. 4A). Among these Fps1 forms, only the form bearing the shortest deletion, which lacks residues 630–649, was able to associate with Rgc2 (Fig. 4C). Because the next larger deletion form of Fps1, which lacks residues 611–649, failed to associate with Rgc2, the 19 residues from 611–629 were implicated as potentially important in this interaction. We reported recently that *C. glabrata* possesses two orthologs of Fps1 (*CgFPS1* and *CgFPS2*), but only *CgFPS1* complements the loss of *S. cerevisiae FPS1* (Beese-Sims et al. 2012). Alignment of Fps1 residues 611–629 with CgFps1 revealed that residues 611–619 (KVQFKSVQR) are perfectly conserved, whereas the remainder of this region is highly divergent. Interestingly, CgFps2 is quite divergent across this region conserved between CgFps1 and ScFps1. Additionally, residues 614–617 (FKSV) have been identified as conserved among various fungal Fps1 orthologs (Pettersson et al. 2005). Therefore, we constructed two additional mutant forms of Fps1: one deleted for residues 611–614 (*fps1-ΔKVQF*) and one in which residues 614–617 were mutated to Ala (*fps1-FKSV*). Both of these mutant forms of Fps1 lost the ability to associate with Rgc2 (Fig. 4D), supporting the conclusion that this region of the Fps1 C terminus is important for interaction with its regulator. Consistent with the conclusion that the interaction between Rgc2 and Fps1 is important for channel activity, the *fps1-FKSV* allele behaved similarly to an *rgc1*Δ *rgc2*Δ double mutant with regard to temperature-dependent cell lysis (Fig. 4E), arsenite resistance (Fig. 4F), and glycerol retention (Fig. 4G). At least the first of these phenotypes was slightly less severe than that of an *fps1*Δ mutant, reflecting the low level of basal channel activity retained in the absence of Rgc1 and Rgc2 function (Beese et al. 2009). Therefore, the *fps1-FKSV* allele encodes a channel that cannot be activated by Rgc2 (or, presumably, Rgc1).

Fps1 possesses a partial PH domain within its C-terminal cytoplasmic domain

As noted above, split PH domains have been found previously to bind partial PH domains in target proteins. In

a second approach to the characterization of the Rgc2-binding site within the C-terminal domain of Fps1, we searched for partial PH domains within this region of Fps1, as described (van Rossum et al. 2005). In brief, we created conceptual chimeras between each region of the tripartite Rgc2 PH domain and the C-terminal domain of Fps1, sliding the Rgc2 sequences through the Fps1 sequence and searching the NCBI conserved domain database with each chimera for a recognizable PH domain. By this approach, we identified a region of the Fps1 C-terminal domain from residues 544 to 581, which, when conceptually fused to the two C-terminal-most parts of the tripartite Rgc2 PH domain, yielded a recognizable PH domain (Supplemental Fig. S3). Therefore, we constructed an additional *fps1* mutant that is deleted for residues 544–581 (*fps1-ΔPHD*). This mutant form was impaired for interaction with Rgc2, as judged by diminished co-IP (Fig. 4D), supporting the hypothesis that this region of Fps1 may form an intermolecular PH domain with the tripartite PH domain of Rgc2. Like the other Rgc2 interaction mutants of *FPS1*, the *fps1-ΔPHD* mutant displayed defects in channel activity, as measured by temperature-dependent cell lysis, arsenite resistance, and glycerol retention (Supplemental Fig. S4).

Hog1 is recruited to Fps1 in response to hyperosmotic shock

As noted above, the Fps1-Δ1 mutant channel was not impaired for the ability to associate with Rgc2. Intriguingly however, Rgc2 failed to dissociate from Fps1-Δ1 in response to hyperosmotic shock (Fig. 5A), suggesting that the N-terminal domain of Fps1 is required for the regulated dissociation of Rgc2. It was reported previously that Hog1 associates in vivo with the N-terminal cytoplasmic domain of Fps1 (Mollapour and Piper 2007). Therefore, we explored the possible role of Hog1 binding to Fps1 in the dissociation of Rgc2 from the Fps1 C-terminal domain. First, we found by co-IP that Hog1 associated weakly with Fps1 in the absence of stress, and the level of this association increased in response to hyperosmotic shock and remained elevated for at least 20 min after shock (Fig. 5B), suggesting that activated Hog1 is recruited to Fps1. This conclusion was supported using bimolecular fluorescence complementation (Lipatova et al. 2012) to visualize the induced association between Hog1 and Fps1 in response to hyperosmotic shock, which unites two halves of a split CFP fluorophore (Fig. 5C). In the presence of 1 M sorbitol, 56.4% of cells displayed a visibly detectable fluorescent signal within 5 min of shock, whereas in the absence of hyperosmotic shock, only 27.6% displayed a detectable signal. Additionally, among those cells with detectable fluorescence, the signal intensity increased 1.8-fold in response to hyperosmotic shock (Supplemental Fig. S5), which may account for the increase in the fraction of cells detected with signal. The observed shift from smooth cell surface fluorescence to punctate surface fluorescence has been reported previously for Fps1 localization (Mollapour and Piper 2007) and may be the consequence of dehydration-induced plasma membrane invaginations (Dupont et al. 2010).

A Hog1-docking site within the N-terminal cytoplasmic domain of Fps1

Hog1 associates with a site within the N-terminal 255 amino acids of Fps1 (Mollapour and Piper 2007). As anticipated, we found that Hog1 associates with wild-type Fps1 and Fps1-C1 but not the N-terminal deletion form Fps1-Δ1 (Fig. 5D). We identified a single potential

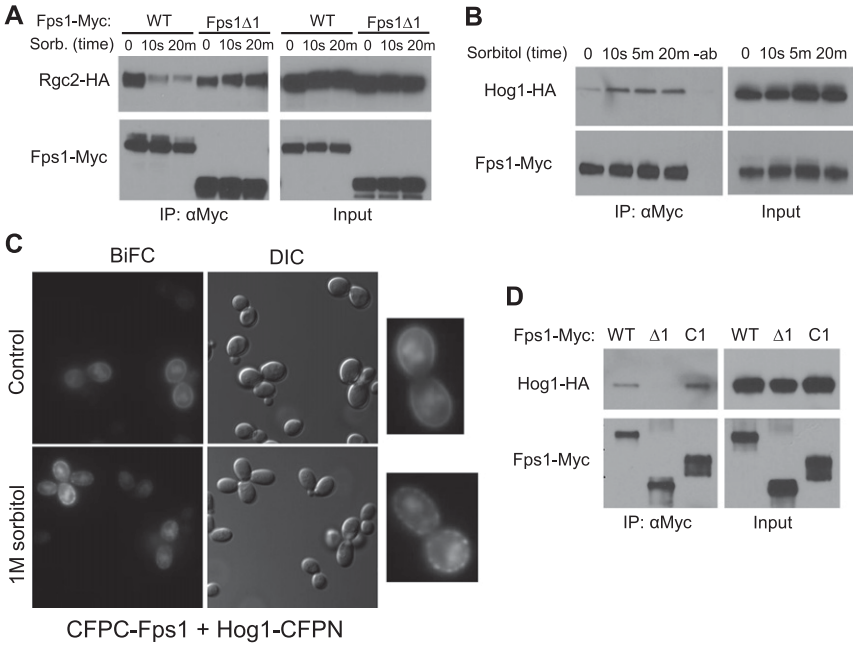

Figure 5. Hog1 is recruited to Fps1 by hyperosmotic shock. (*A*) Rgc2 is stabilized on an N-terminal truncation mutant of Fps1. Co-IP of Rgc2 with wild-type Fps1 or Fps1Δ1. Fps1-Myc was coexpressed in *fps1*Δ cells (DL3226) with Rgc2-HA and treated as in Figure 1B. (*B*) Hog1 is recruited to Fps1 by hyperosmotic shock. Co-IP of Fps1-Myc coexpressed with Hog1-HA in wild-type cells (DL3187). (−ab) No α-Myc antibody. (*C*) Bimolecular fluorescence complementation (BiFC) of CFPC-Fps1 with Hog1-CFPN. Representative micrographs of wild-type cells coexpressing Fps1 and Hog1 (each tagged with half of the CFP fluorophore) that were exposed to hyperosmotic shock with 1 M sorbitol for 5 min prior to visualization. Enlarged images of representative fluorescent cells are at the *right*. (*D*) Co-IP of N-terminal or C-terminal domain truncation of Fps1-Myc coexpressed with Hog1-HA in *fps1*Δ cells (DL3226) in the absence of osmotic shock.

MAPK-docking site (D motif) (Sharrocks et al. 2000; Zhang et al. 2003) within this region of Fps1 (residues 213–220). Point mutations across this region result in constitutive channel activity (Tamás et al. 2003). Therefore, we ablated this potential docking site by converting residues 218 and 220 to Ala (Fps1-IV/AA). This mutant form of Fps1 displayed a constitutive channel character similar to the *FPS1-Δ1* form, as judged by increased arsenite sensitivity (Fig. 6A) and elevated glycerol efflux (Fig. 6B). Hog1 failed to associate with Fps1-IV/AA either under basal conditions or in response to hyperosmotic shock (Fig. 6C), thus supporting the conclusion that these residues are part of a Hog1-docking site. As was the case for the interaction between Rgc2 and Fps1-Δ1, Rgc2 failed to dissociate from Fps1-IV/AA in response to hyperosmotic shock (Fig. 6D). Taken together, these results indicate that a key regulatory role of the Fps1 N-terminal domain is to serve as a scaffold for recruitment of active Hog1, an event that appears to be required for the dissociation of Rgc2 under conditions of hyperosmotic shock.

The above conclusion was also supported by the finding that the phosphorylation-induced bandshift of Rgc2 is partially dependent on the ability of both Rgc2 and Hog1 to associate with Fps1 (Fig. 6E). We demonstrated previously that Rgc2 is hyperphosphorylated in response to hyperosmotic shock in a manner partially dependent on Hog1 (Beese et al. 2009). Figure 6E shows that, in the absence of either the Hog1-docking site (*FPS1-IV/AA*) or the Rgc2-binding site (*fps1-FKSV*) of Fps1, there is a reduction in the hyperosmotic shock-induced Rgc2 bandshift. These results suggest that both Rgc2 and Hog1 must reside on their respective domains of Fps1 for efficient hyperosmotic shock-induced Rgc2 phosphorylation to occur.

Discussion

Glycerol serves as a compatible solute in *S. cerevisiae* and other yeasts, allowing cells to respond quickly to changes in external osmolarity. The Fps1 glycerol channel is a key component in the control of cytoplasmic glycerol concentration. Although Fps1 is known to close under conditions of hyperosmotic shock and open in response to hypo-osmotic shock (Tamás et al. 1999), the mechanism by which Fps1 function is modulated by changes in osmolarity is not understood. Previous studies have implicated both the MAPK Hog1 (Tamás et al. 1999; Hohmann 2009) and the redundant PH domain proteins Rgc1 and Rgc2 (Beese et al. 2009) in the regulation of Fps1. In this study, we demonstrate that Hog1 induces the closure of Fps1 under conditions of hyperosmotic shock through phos-

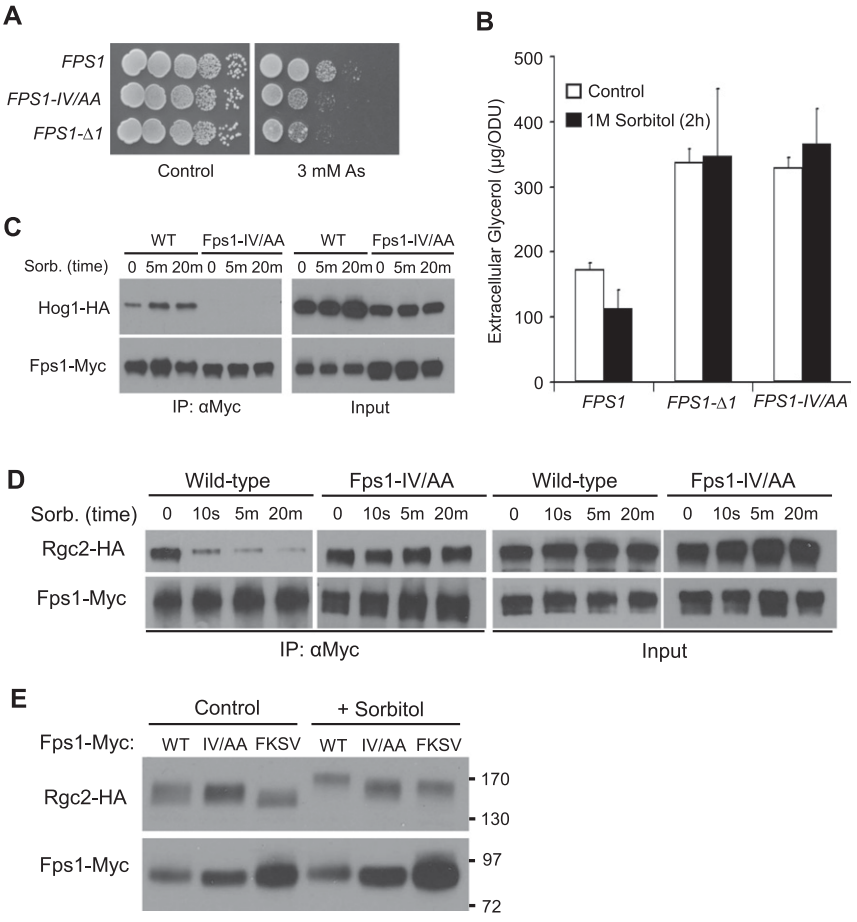

Figure 6. A Hog1-docking site within the N-terminal domain of Fps1. (*A*) An *FPS1-I218A, V220A* (*FPS1-IV/AA*) mutant is hypersensitive to arsenite. Cultures of an *fps1*Δ strain (DL3226), transformed with plasmids expressing the indicated *FPS1* allele, were spotted onto YEPD plates with or without 3 mM arsenite and incubated for 3 d at 30°C. (*B*) The *FPS1-IV/AA* mutant releases excess glycerol into the medium. Glycerol was measured in the culture medium from the same strains with or without 1 M sorbitol for 2 h. (*C*) Hog1 fails to bind to the Fps1-IV/AA protein. Co-IP of Hog1-HA with the indicated form of Fps1-Myc coexpressed in DL3226. Cells were treated as in Figure 1B. (*D*) Rgc2 is stabilized on the Fps1-IV/AA channel. Co-IP of Rgc2-HA with the indicated form of Fps1-Myc coexpressed in DL3226. (*E*) Phosphorylation of Rgc2 in response to hyperosmotic shock is diminished in mutant forms of Fps1 that cannot bind Hog1 or Rgc2. Rgc2-HA was coexpressed with the indicated form of Fps1-Myc in DL3226. Cultures were exposed to hyperosmotic shock with 1.8 M sorbitol for 5 min. Samples for this panel were resolved to show Rgc2 bandshifts. Molecular mass markers are shown at the *right*.

phorylation and eviction of Rgc2 (and presumably Rgc1) from the Fps1 C-terminal cytoplasmic domain (see the model in Fig. 7).

Rgc2 maintains Fps1 in an open state

We found that, under unstressed conditions, Rgc2 binds to the C-terminal cytoplasmic domain of Fps1 and that this association is responsible for maintaining the channel in an open state. Several observations underscore this conclusion. First, hyperosmotic shock resulted in rapid, Hog1-dependent eviction of Rgc2 from Fps1. Second, Hog1 phosphorylation site mutant forms of Rgc2 (e.g., *RGC2-7A*) were stabilized on Fps1 and resulted in a constitutively open channel phenotype. Third, mutant forms of Rgc2 that could not bind to Fps1 (e.g., PH domain mutants) were defective for Fps1 channel activity. Fourth, mutant forms of Fps1 that could not bind to Rgc2 (e.g., *fps1-FKSV*) were also defective for channel activity.

We identified two regions of the Fps1 C-terminal domain that were important for Rgc2 association. Identification of one interaction region, which spans Fps1 residues 544–581, was based on both our finding that the tripartite PH domain of Rgc2 is required for its interaction with Fps1 and the premise that split PH domains are known to form intermolecular PH domains with target proteins (van Rossum et al. 2005; Yan et al. 2005). We identified this interaction region by conceptually "threading" different parts of the Rgc2 PH domain through the Fps1 C terminus to create a chimeric PH domain, suggesting that Fps1 possesses a partial PH domain. The other region of Fps1 important for Rgc2 interaction encompasses residues 611–617. This site was identified through deletion-mapping experiments and homology with Fps1 orthologs from other fungal species. However, this interaction site does not appear to be within a PH domain-like sequence. Rgc2 may therefore have multiple interaction sites within the C-terminal domain of Fps1.

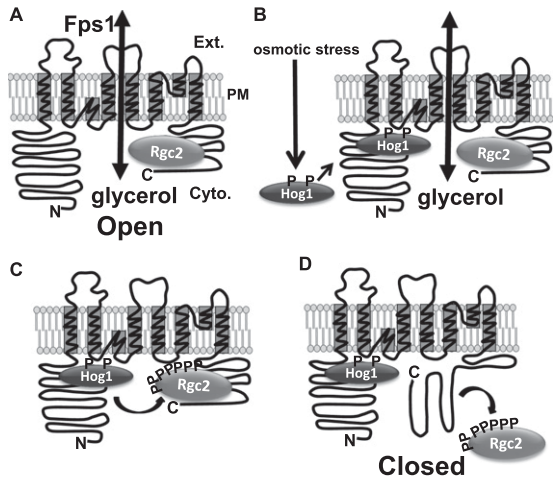

Figure 7. Model for the regulation of Fps1 by Hog1 in response to hyperosmotic shock. (*A*) Under nonstress conditions, Rgc2 is associated with the C-terminal domain of Fps1, maintaining it in an open state. (*B*) Hog1 is activated in response to hyperosmotic shock and recruited to the N-terminal domain of Fps1. Hog1 uses Fps1 as a platform from which to phosphorylate Rgc2 (*C*), inducing its eviction from the C-terminal domain and thereby allowing channel closure (*D*).

We found that mutation of the Rgc2-binding sites within the C terminus of Fps1 resulted in channel closure and the accumulation of glycerol in the absence of hyperosmotic shock. Although numerous C-terminal and N-terminal mutant forms of Fps1 have been identified with open channel phenotypes (Tamás et al. 2003; Hedfalk et al. 2004), we are not aware of any previous reports of Fps1 mutants across these regions with closed channel phenotypes.

It is not clear how the presence of Rgc2 (or Rgc1) on the C terminus of Fps1 exerts its impact on channel activity. Removal of the Fps1 C-terminal domain was shown previously by Hohmann and colleagues (Hedfalk et al. 2004) to result in an unregulated channel, prompting the suggestion that this domain functions to interfere with glycerol flux through the channel. These investigators identified a region near the last transmembrane domain of Fps1, comprising residues 535–546, that is important for channel closure and proposed that it may dip into the membrane to interact with another part of the channel. If this is the case, Rgc2-binding to Fps1 at the partial PH domain immediately adjacent to this region (residues 544–581) may hold it in a conformation that prevents such interactions.

Hog1 displaces Rgc2 from Fps1 through multiple phosphorylation events

We demonstrated previously that Rgc2 becomes hyperphosphorylated in response to osmotic shock in a manner that is partially dependent on Hog1. Here we observed that, in a *hog1*Δ mutant, Rgc2 failed to be evicted from Fps1 in response to hyperosmotic shock. Therefore, we identified by mass spectrometric analyses 30 phosphorylation sites on Rgc2, seven of which reside within MAPK phosphorylation motifs (S/T-P). Three of these phosphorylation sites were confirmed to be Hog1 target sites based on both their induction by hyperosmotic shock and their inhibition by a Hog1-specific inhibitor. The remaining four sites could not be assigned unambiguously as Hog1 sites. A mutant form of Rgc2 that blocks the three confirmed Hog1 phosphorylation sites (*RGC2-3A*) was partially stabilized on Fps1 in response to osmotic shock. However, a mutant form that blocks these sites together with an additional four phosphorylation sites suspected to be Hog1 target sites (*RGC2-7A*) was almost completely stabilized on Fps1 in response to osmotic shock. We conclude from these results that Hog1 drives the closure of Fps1 by phosphorylating and evicting Rgc2 (and presumably Rgc1) from the Fps1 channel (Fig. 7).

The PH domain of Rgc2 resides within its central region. However, most of the 30 identified phosphorylation sites within Rgc2 are clustered within its C-terminal domain, suggesting that this region may be key for Rgc2 regulation. Five of the seven confirmed and suspected Hog1 phosphorylation sites in Rgc2 are located within this domain, and two are located within the N-terminal

domain. The PH domain is notably devoid of Hog1 phosphorylation sites. However, it should be noted that, because our MS results did not cover the entire Rgc2 sequence, we cannot rule out the possibility that additional S/T-P motifs are phosphorylated by Hog1.

Hog1 uses the Fps1 N-terminal domain as a platform from which to phosphorylate Rgc2

We made the intriguing finding that Rgc2 fails to be evicted in response to hyperosmotic shock from the C-terminal domain of a mutant form of Fps1 lacking its N-terminal cytoplasmic domain. A previous report indicated that Hog1 binds to the N-terminal domain of Fps1 (Mollapour and Piper 2007), and we extended this finding here with the demonstration that Hog1 is recruited to a MAPK-docking site within the Fps1 N-terminal domain when activated by hyperosmotic shock. Mutations across this region of Fps1 are known to display constitutive channel phenotypes (Tamás et al. 1999). Moreover, we found that Hog1 must bind to this site to phosphorylate Rgc2 efficiently and evict it from the Fps1 C terminus in response to hyperosmotic shock (Fig. 7). Two results support this conclusion. First, Rgc2 was stabilized on a mutant form of Fps1 that lacks its Hog1-docking site (Fps1-IV/AA) (Fig. 6C). Second, Rgc2 was not fully phosphorylated in the *FPS1-IV/AA* mutant in response to hyperosmotic shock (Fig. 6D).

Thus, we identified three types of mutations that stabilize Rgc2 on the C terminus of Fps1 and result in constitutively open channel phenotypes. These are (1) a *hog1*Δ mutant, (2) Hog1 phosphorylation site mutants of Rgc2, and (3) a Hog1-docking site mutant of Fps1. These findings collectively support the conclusion that Hog1 drives the closure of Fps1 in response to hyperosmotic shock by using the N-terminal domain of Fps1 as a platform from which to phosphorylate and evict Rgc2 from the Fps1 C-terminal domain.

It appears from our data and those of Mollapour and Piper (2007) that some Hog1 resides on Fps1 even in the absence of hyperosmotic stress. Perhaps Hog1 engages in priming phosphorylations of Rgc2 bound to Fps1. Such priming phosphorylations might facilitate the rapid eviction of Rgc2 under stress conditions. Alternatively, some Hog1 may be poised on Fps1 to catalyze Fps1 phosphorylation in response to acetic acid stress. In this regard, it is interesting to note that the mechanism by which Hog1 controls Fps1 in response to hyperosmotic shock is very different from the mechanism it uses in response to acetic acid stress. In the latter case, Hog1 phosphorylates Fps1 on residues within its N-terminal and C-terminal domains, which triggers its ubiquitin-mediated endocytosis and degradation (Mollapour and Piper 2007). It should be noted that there is no evidence that Hog1 phosphorylates Fps1 in response to hyperosmotic shock (Ahmadpour et al. 2013), and our own phosphoproteomic analysis of Fps1 suggests that Hog1 does not target the channel directly under these conditions (W Reiter, unpubl.). One explanation for the different behavior of Hog1 in response to these stresses is that hyperosmotic shock is transient and resolved by the production and retention of glycerol, whereas acetic acid stress is likely to pose a longer-term problem. Therefore, the former is amenable to a solution that involves a reversible change to Fps1, whereas the latter may be best addressed by destruction of Fps1. An interesting unresolved issue concerns how active Hog1 discriminates between these two mechanisms. There may be additional factors that are differentially recruited to Fps1 under various Hog1-activating conditions to control its fate. Alternatively, Fps1 may adopt different conformations in response to these different stresses that would impact the ability of Hog1 to phosphorylate either Fps1 or its resident, Rgc2.

Very little is known about the mechanisms by which aquaglyceroporins are regulated in other fungal species. However, the *FPS1* gene from the human pathogen *C. glabrata* complements the temperature-dependent cell lysis defect of an *S. cerevisiae fps1*Δ mutant (Beese-Sims et al. 2012). Additionally, the *C. glabrata RGC2* gene complements the cell lysis defect of an *S. cerevisiae rgc1*Δ *rgc2*Δ mutant, suggesting that the Rgc–Fps regulatory arrangement is conserved between these two species and might be exploited as a drug target (Beese-Sims et al. 2012). However, no phosphoproteomic data are available for *C. glabrata* Rgc2, and only two of the seven phosphorylated S/T-P motifs identified in *S. cerevisiae* Rgc2 are conserved in *C. glabrata* Rgc2 (S75 and S827). Thus, it is not yet clear whether Hog1 plays the same regulatory role for the *C. glabrata* glycerol channel as for *S. cerevisiae* Fps1.

Similarly, little is known about the molecular regulation of aquaglyceroporins in animals, where efforts have been focused on control of their expression (Rojek et al. 2008; Maeda 2012). However, a recent report on the *Leishmania* aquaglyceroporin AQP1, the major entry port for antimony-containing drugs, revealed that a MAPK can phosphorylate this protein within a cytoplasmic loop (Mandal et al. 2012). This modification causes the stabilization of AQP1 and its delocalization from the flagellum to the entire cell surface, resulting in increased transport activity.

In any case, it is likely that the mechanism described here for regulation of Fps1 is restricted to fungal species because key components are unique to fungi—the N-terminal and C-terminal cytoplasmic extensions of Fps1 are unusual among aquaglyceroporins and appear to be restricted to fungal species, as are homologs of the Rgc proteins. The specificity of this mechanism to fungi provides an opportunity for antifungal drug development.

Materials and methods

Strains, growth conditions, transformations, and gene replacements

The *S. cerevisiae* strains used in this study were all derived from the Research Genetics background S288c (Research Genetics, Inc.) and are listed in Supplemental Table S1. Yeast cultures were grown in YPD (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) or SD (0.67% yeast nitrogen base, 2% glucose) supplemented with the appropriate nutrients to select for plasmids. Yeast cultures were transformed according to Gietz et al. (1995).

Chromosomal integration of *RGC2* alleles in an *rgc1Δ::KanMX RGC2* strain were carried out by a two-step process. First, the *RGC2*-coding region of the *rgc1Δ::KanMX RGC2* strain (DL3186) was replaced with the *URA3* gene by transformation with PCR-generated *URA3* flanked on either side by 40 nucleotides of 5′ and 3′ noncoding *RGC2* sequence immediately adjacent to the coding sequence. This was done to ensure that mutant alleles of *RGC2* integrated in the second step would not recombine with endogenous *RGC2*-coding sequence. The resulting strain, *rgc1Δ::KanMX rgc2Δ::URA3*, was designated DL4046. Second, cassettes were constructed in integration vector pAG32 (*hphMX4*) (Goldstein and McCusker 1999) to contain mutant alleles of *RGC2* tagged at its C terminus with the Flag epitope ligated at its 3′ end to the *hphMX4* gene, which confers resistance to hygromycin B. Linear PCR products were amplified from the engineered cassettes and transformed into DL4046. Integrants were selected on plates containing hygromycin B and tested for loss of Ura^+, and gene replacement was confirmed by PCR analysis across the integration junctions. The correct sequences of mutant *RGC2* alleles were confirmed by genomic DNA sequence analysis.

Plasmid construction

The plasmids used in this study are listed in Supplemental Table S2 and were constructed as described in the Supplemental Material.

Co-IP and immunoblot analysis

Cultures for co-IP experiments with Fps1-Myc and Rgc2-HA or Hog1-HA were grown to mid-log phase in selective medium and starved for methionine for 2 h to induce expression of Rgc2-HA and Fps1-Myc, which were expressed under the control of the conditional *MET25* promoter. For osmotic stress experiments, cultures were shocked by adding 3 M sorbitol to a final concentration of 1.8 M. Protein extraction and co-IPs were carried out as described previously (Kamada et al. 1995), except that 0.5% Triton X-100 was included in the lysis buffer. Extracts (100 μg of protein) were exposed to 1 μg of mouse monoclonal α-Myc antibody (9E10, Pierce) for 1 h at 4°C and precipitated with either protein A affinity beads (for Rgc2-HA and Fps1-Myc; Sigma) or protein G affinity beads (for Hog1-HA and Fps1-Myc; Sigma) for 1 h at 4°C. Samples were washed with immunoprecipitation buffer three times and boiled in SDS-PAGE buffer. Proteins were separated by SDS-PAGE (7.5% gels) followed by immunoblot analysis using α-Myc antibody (9E10, Pierce) or α-HA (16B12, Covance) at a dilution of 1:10,000. Secondary antibodies (goat anti-mouse; Amersham) were used at a dilution of 1:10,000.

Bimolecular florescence complementation

Wild-type haploid cells were transformed with plasmids pRS413-*CFPC-FPS1* and pRS415-*HOG1-CFPN*, which express the indicated fusion proteins. Transformants were grown overnight in SD medium, diluted in YPD for growth to mid-log phase, exposed to hyperosmotic shock by centrifugation, and resuspended in SD medium with or without 1 M sorbitol immediately before visualization. Cells were visualized with a Zeiss Axio Observer Z1 with a 100× objective fitted with a CFP filter.

Measurement of β-galactosidase activity and intracellular/extracellular glycerol concentrations

Measurement of β-galactosidase activity from *GPD1-lacZ* expression experiments was carried out as described in Zhao et al. (1998). Intracellular glycerol concentrations were measured in whole cells grown in YPD or hyperosmotically shocked and centrifuged briefly to remove the culture supernatant. Extracellular glycerol concentrations were measured from the culture supernatant. For these experiments, cells were centrifuged and resuspended in fresh medium prior to hyperosmotic shock to eliminate glycerol present in the medium prior to the shock. Enzymatic assays for glycerol were carried out using a kit from Boehringer Mannheim and normalized to A_{600} of the initial culture.

MS

Mass spectrometric analyses of phosphopeptide sites within Rgc2 and Rgc1 were carried out as described in the Supplemental Material.

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