Protein Interactions of the Mechanosensory Proteins Wsc2 and Wsc3 for Stress Resistance in Saccharomyces cerevisiae

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ABSTRACT Antifungal drug discovery and design is very challenging because of the considerable similarities in genetic features and metabolic pathways between fungi and humans. However, cell wall composition represents a notable point of divergence. Therefore, a research strategy was designed to improve our understanding of the mechanisms for maintaining fungal cell wall integrity, and to identify potential targets for new drugs that modulate the underlying protein-protein interactions in Saccharomyces cerevisiae. This study defines roles for Wsc2p and Wsc3p and their interacting protein partners in the cell wall integrity signaling and cell survival mechanisms that respond to treatments with fluconazole and hydrogen peroxide. By combined genetic and biochemical approaches, we report the discovery of 12 novel protein interactors of Wsc2p and Wsc3p. Of these, Wsc2p interacting partners Gtt1p and Yck2p, have opposing roles in the resistance and sensitivity to fluconazole treatments respectively. The interaction of Wsc2p with Ras2p was confirmed by iMYTH and IP-MS approaches and is shown to play a dominant role in response to oxidative stress induced by hydrogen peroxide. Consistent with an earlier study, Ras2p was also identified as an interacting partner of Wsc1p and Mid2p cell wall integrity signaling proteins. Collectively, this study expands the interaction networks of the mechanosensory proteins of the Cell Wall Integrity pathway.

KEYWORDS mechanosensory proteins cell wall integrity pathway stress response protein-protein interactions

The frequency of systemic infections caused by opportunistic fungal pathogens of the normal endogenous flora is increasing worldwide (Van Thiel et al. 2012; Boral et al. 2018). Candida auris is rapidly emerging as a cause of hospital-acquired multidrug-resistant fungal infections at a global level (Chowdhary et al. 2017), while drug-resistant strains of Candida glabrata are also becoming increasingly responsible for systemic infections of immunocompromised patients (Rosenwald et al. 2016). Similarly, fungal infections with Aspergillus fumigatus and Cryptococcus neoformans can be acquired from non-endogenous host surroundings (Badiee and Hashemizadeh 2014). Infections in patients with severe immunological impairment can have fatal consequences due to the development of resistance by these fungi to the limited number of therapeutic antifungal drugs currently available (Roemer and Krysan 2014; Sanglard 2016). Therefore, there is an urgent need for new therapeutic strategies to address drug resistance in fungal infections.
Multiple forms of environmental stresses that compromise cell wall structure or function can activate the MAPK cascade underpinning the Cell Wall Integrity (CWI) pathway. The initial steps for activating the CWI response occur through interactions mediated by the N-terminal regions of the \textit{Wsc1p}, \textit{Wsc2p}, \textit{Wsc3}, \textit{Mid2p} and \textit{Mtl1p} mechanosensory proteins with the cell wall (Staehle and Heinisch 2007; Heinisch et al. 2010; Kock et al. 2016). The binding of the GDP-GTP Exchange Factor (GEF), \textit{Rom2p}, to the cytoplasmic C-terminal ends of these proteins and the activation of the small GTPase \textit{Rho1p} are integral steps in CWI pathway activation (Ozaki et al. 1996; Philip and Levin 2001). However, proteins acting upstream of \textit{Rom2p} recruitment in this activation step have yet to be identified.

The MAPK cascade directed by the CWI pathway is regulated by protein kinase C1, \textit{Pkc1p} (Levin 2005; Fuchs and Mylonakis 2009) and a stress inducible phosphatase, \textit{Sdp1p} (Hahn and Thiele 2002). \textit{Rom2p} binding to the \textit{Wsc1p} C-terminal tail requires dephosphorylation of specific serine residues of \textit{Wsc1p} within a serine-rich motif, specifically S319, S320, S322, S323, (Vay et al. 2004) but the specific enzymes that phosphorylate and dephosphorylate these residues and how they interact with \textit{Rom2p} are uncertain. In addition to chemical or genetic perturbation of the cell membrane, the CWI pathway is stimulated by a wide variety of other stresses including nutrient starvation, hypo-osmotic shock, heat shock, and environmental conditions that perturb cell integrity functions (Kamada et al. 1993; Fuchs and Mylonakis 2009). Readouts of the CWI pathway include the enhanced transcription of genes encoding stress proteins and wall components, altered translational regulation, and the modulation of growth processes (Kamada et al. 1995; Gray et al. 1997; de Nobel et al. 2000; Zu et al. 2001; Rodríguez-Quiones et al. 2008; Rodríguez-Quiones and Rodríguez-Medina 2009; Rivera-Ruiz et al. 2010; Rodicio and Heinisch 2010; Pagán-Mercado et al. 2012; Yurko et al. 2017; Leskoske et al. 2018). The CWI pathway also controls genes involved in other biological processes such as regulation of cytoskeleton polarization (de Nobel et al. 2000), the activity of cell wall biosynthetic enzymes (Levin 2011), polarized cell growth (Zarov et al. 1996), and the control of target of rapamycin complex 1 (TORC1) (Petkova et al. 2010) and TORC2 complexes (Leskoske et al. 2018).

In the budding yeast \textit{Saccharomyces cerevisiae}, mechanosensory proteins represented by the Wsc-family (\textit{Wsc1p}, \textit{Wsc2p}, and \textit{Wsc3p}) and the Mid-family (\textit{Mid2p} and its homolog \textit{Mtl1p}), are responsible for detecting and transmitting cell wall, nutritional, and environmental stress cues through the CWI pathway (Verna et al. 1997). These proteins share amino acid sequence identity of approximately 30% between the Wsc-family members and 50% between the Mid-family members. The Wsc-family proteins are distinguished by the presence of a cysteine-rich domain at the N-terminal region which is covalently attached to cell wall glycoproteins via glucan linkages. The presence of a cysteine-rich domain at the N-terminal region which is covalently attached to cell wall glycoproteins via glucan linkages. The Wsc-family proteins are reported to elicit different responses to stress conditions. Therefore, it is proposed that multiple, and possibly redundant, survival pathways become activated by different types of stresses (Santiago-Cartagena et al. 2019).

There is a high level of conservation of mechanosensory proteins of the CWI pathway among fungal pathogens. Putative genes encoding homologs of the \textit{S. cerevisiae} Wsc-family (AfWsc1-3) and Mid2-family (AfMida) were previously identified in \textit{Aspergillus fumigatus} by BLAST analysis (Dichtl et al. 2012; Dichtl et al. 2016). In \textit{A. fumigatus}, mutants of components of the CWI pathway show the same sensitivity to compounds typically used to characterize the CWI pathway in \textit{S. cerevisiae} (Valiante et al. 2015). The \textit{Candida albicans} CWI pathway is activated by stresses similarly to \textit{S. cerevisiae}, with \textit{C. albicans} encoding CaWsc1p and CaWsc2p homologous to \textit{Wsc1p} and \textit{Wsc2p} of \textit{S. cerevisiae}, respectively (Dichtl et al. 2012). The \textit{Cryptococcus neoformans} MAPK module is composed of three members known as MAPKKK CnBck1, MAPKK CnMkk2, and MAPK CnMpk1 which are similar to \textit{Bck1p}, \textit{Mkk2p} and \textit{Mpk1p} of \textit{S. cerevisiae}, respectively. In humans, these proteins are non-existent or have evolved different functions making them potentially good therapeutic targets for antifungal agents. Due to the high level of homology among mechanosensory proteins and the components of MAPK module, it has become evident that other fungi including human pathogens share a CWI signal transduction mechanism similar to \textit{S. cerevisiae}.

\textit{Wsc2p} and \textit{Wsc3p} are reported to have redundant functions. A \textit{wsc2Δ} null mutant strain exhibits reduced competitive fitness, consistent with a basic housekeeping function in cell biology (Wilkinson et al. 2010). A \textit{wsc3Δ} null mutant strain was reported to show increased sensitivity to arsenate, a response that was suppressed by sorbitol, suggesting that \textit{Wsc3p} responds to cell wall stress treatments (Matia-González and Rodriguez-Gabriel 2011). Compared with a wildtype control, caspofungin treatment of \textit{wsc2Δ} and \textit{wsc3Δ} strains induces the increased accumulation of hyper-phosphorylated \textit{Shl2p}, the read-out for the CWI pathway activation (Remison-Martin et al. 2003). Double and triple mutants of \textit{wsc1Δ} combined with \textit{wsc2Δ}, \textit{wsc3Δ}, or \textit{wsc2Δwsc3Δ} mutation have been evaluated for growth phenotypes by Verna et al. (1997). Under normal growth conditions on synthetic defined (SC) medium at 28°C, none of the single mutants \textit{wsc1Δ}, \textit{wsc2Δ}, or \textit{wsc3Δ} were thermosensitive, while specific combinations of these mutants (i.e., \textit{wsc1Δwsc2Δ}, \textit{wsc1Δwsc3Δ}, and \textit{wsc1Δwsc2Δwsc3Δ}) acquired the thermosensitive phenotype, supporting the notion that the WSC genes share redundant functions.

In a previously published study from our laboratory, we reported novel protein-protein interactions (PPIs) of \textit{Wsc1p} and \textit{Mid2p} (Santiago-Cartagena et al. 2019). Here, we aim to expand the known interactome map of \textit{Wsc2p} and \textit{Wsc3p} by identifying novel PPIs using the integrated Membrane Yeast Two Hybrid (iMYTH) screening method (Fetchko et al. 2003; Fetchko and Staglar 2004; Paumi et al. 2007; Snider et al. 2010) and to score the newly identified factors for function in the regulation of growth and the activation of the CWI pathway in response to treatments with caspofungin (CS), fluconazole (FCZ), and hydrogen peroxide (H$_2$O$_2$). The results reported here provide new insight into the CWI signal transduction mechanism active in \textit{S. cerevisiae} and other fungi.

**MATERIALS AND METHODS**

**Strains, transformations and growth conditions**

To create iMYTH bait strains, iMYTH L2 or L3 cassettes containing the 35-40 bases of homology near the C-terminal end of the \textit{WSC2} or
using the NubG forward internal primer (Table S2). In-house software DNA that could be re-screened for activity. 259 cDNA clones that ampicillin and grown for 2 days at 37°C resulting hybrid genes were transformed into MYTH reporter strains struct of the bait strain was veri

The iMYTH library screens were conducted as described by Snider et al. (1994) in the BY4742 genetic background (Open Biosystems). The wild type strains, BY4741 and BY4742, were obtained from ATCC. See Supplementary Table S1 for yeast strains and Supplementary Table S2 for DNA primers used in this study.

Library screening
iMYTH library screens were conducted as described by Snider et al. (2010). Putative positive transformants obtained with 40-80 µg of NubG-X cDNA prey library were selected on BD-Trays (Beckton-Dickinson) containing Synthetic Dropout medium without Tryp
tophan, Adenine, and Histidine (SD-WAH) or Synthetic Dropout medium without Tryptophan and Histidine (SD-WH) incubated at 30°C for 3-4 days to select for markers TRP1 present on the vector DNA, or ADE2 and HIS3 that are integrated into the genome as part of the iMYTH reporter system. For the Optimized Large Scale Transformation, 20 ml of bait strain culture in 2X Yeast Peptone hydrolysate plus Dextrose broth medium (YPD) was transformed with 40-80 µg of NubG-X cDNA prey library.

Plasmid recovery and sequencing
Single colonies obtained from the large-scale iMYTH transformation of bait strains were picked and diluted into 50 µl sterile 0.9% NaCl solution. Afterward, 2.5 µl of re-suspended cells were plated onto SD-WAH or SD-WH plates containing X-Gal and grown for 1-3 days at 30°C to screen for trans-activation of the lacZ reporter gene. Blue colonies were inoculated into SD-W in 96-well blocks and grown for 2 days at 30°C. The pellets were re-suspended with 125 µl of Lysis solution [β-mercaptoethanol, Solution A (1M Sorbitol, 0.1 M Sodium Citrate, 60 mM EDTA) and Zymolase Solution (Zymolase powder, 1 M Sorbitol)] and treated for 2 hr at 37°C. Plasmid DNA from these transformants was isolated using Nucleospin 96-well miniprep kits according to the manufacturer’s protocol.

Competent DH5α E. coli was transformed with the plasmids recovered from the yeast minipreps and plated on LB agar with 100 µg/ml ampicillin. For high-throughput transformation, 96-well plates and 96-well blocks were used. For E. coli minipreps, single colonies were inoculated in Terrific Broth containing 100 µg/ml ampicillin and grown for 2 days at 37°C. The recovered plasmids were purified using Nucleospin columns and the plasmid DNA sequenced using the NubG forward internal primer (Table S2). In-house software (developed at the University of Toronto) was used for large-scale BLAST analysis and identification of yeast protein sequences.

In the primary screen, a total of 885 colonies for Wsc2p and 406 colonies for Wsc3p containing cDNAs encoding putative inter

actors were picked from the transformation media. From 233 colonies for Wsc2p and 178 colonies for Wsc3p, 280 cultures yielded plasmid DNA that could be re-screened for activity. 259 cDNA clones that were identified by Sanger sequencing represented 58 different proteins that underwent Bait Dependency Tests. 15 out the 58 cDNAs passed the Bait Dependency Tests.

Bait dependency test
Bait Dependency Tests (BDT) were performed to validate iMYTH positive clones (Snider et al. 2010). The purified prey plasmids were transformed into the yeast bait strains Wsc2p THY L2, Wsc3p THY L3, and A0286 or Wsc2p L40 L3, Wsc3p L40 L3 and A0287. Note that A0286 and A0287 correspond to ‘negative control’ reporter strains stably expressing artificial bait construct, in THY.A4 and L40 background, respectively. The resulting transformations were plated onto SD-W media and incubated at 30°C for 3-4 days. The transformant colonies were selected in triplicate and plated onto SD-WA + X-Gal or SD-WH + X-Gal. Preys that caused growth and blue coloration in a bait strain, but not in control bait strain, were considered to be specific or true interactors (Pavini et al. 2007; Snider et al. 2010). The specific interactors for the mechanosensory proteins were classified according to their biological process derived from SGD (Cherry et al., 2012). The Cytoscape, version 3.2.1, software (Shannon et al. 2003) was used to generate protein interactome maps.

Western blot assay for activation of the CWI pathway under stress conditions
To induce cell wall stress, 75 ng/ml of Ca was added to 25 ml of culture at OD600~0.7-0.9. For induction of oxidative stress, hydrogen peroxide (H2O2) was added at a final concentration of 1 mM to 25 ml of culture at OD600~0.7-0.9. Plasma membrane stress was induced with 100 µM FCZ added to cultures under similar conditions. All cultures were incubated at 27°C for 1 hr before harvesting. In all subsequent assays, the results obtained with the experimental cultures were compared to yeast cultures without any stress treatment.

For protein recovery, yeast cultures (25 ml) were centrifuged for 5 min at 3,838 × g and then transferred to 1.5 ml microcentrifuge tubes. The cells were washed in ice cold distilled water, centrifuged at 17,530 × g for 3 min at 4°C and pellets suspended in 0.5 ml lysis buffer (50 mM Tris-Cl, pH 7.5, 10% glycerol, 1% TritonX-100, 0.1% SDS, 150 mM NaCl, 5 mM EDTA) supplemented with 5X Protease Inhibitor Cocktail (PIC) (Roche), 1X phosphatase inhibitor cocktail (II and III, Sigma) and 5X PMSF (10 mM). The cells were mechanically disrupted by the addition of approximately 200 µl of sterile glass beads followed by five sets of alternate vortexing at full speed for 45 sec followed by 3 min incubation on ice. After disruption, the contents in the tubes were centrifuged at 17,530 × g for 10 min at 4°C and the clarified protein supernatants transferred to new pre-chilled 1.5 ml microtubes. An aliquot of each extract was used for determining protein concentration using the DC Protein Assay (Bio-Rad). Afterward, 50 µg of total protein was denatured by heating at 95°C in the presence of a 4X Laemmli dye solution and 5% per volume β-mercaptoethanol for 10 min and then separated in 10% polyacrylamide gels by SDS-PAGE. The proteins in the gels were then transferred to nitrocellulose membranes (BioRad) using a constant electrical current for 90 min at 4°C. The membranes were probed with the following primary antibodies: anti-human phospho-p44/42 MAPK rabbit mononal (Cell Signaling Technology) and yeast Phosphoglycerate kinase (Pyk1p) mouse monoclonal (Molecular Probes, Invitrogen) at a 1:1,000 dilution. The primary antibodies were then incubated in Odyssey blocking buffer (LI-COR) and incubated at room temperature (RT) for 1 hr. The membranes were washed twice with 1X PBS/ 0.2% Tween20 for 10 min per wash. The secondary antibodies used were Goat anti-Rabbit IRDye 800CW (1:10,000) LI-COR (green) and Goat
anti-Mouse IRDye 680LT (1:10,000) LI-COR (red). The membranes were incubated with secondary antibodies for 30 min at RT and washed as described above. 1X PBS was used to wash the membranes before scanning with an Odyssey CLX Infrared Fluorescent Imaging System (LI-COR). Numerical values expressed in Figures 5A-5C represented as the fold-change of the P-Slt2p band intensity in each experimental sample, adjusted to the P-Slt2p band intensity of wild type control sample (WT), each normalized against the intensity of Pgk1p. For statistical analysis by the Student T Test, a minimum of 3 replicates were conducted per experiment and each data point shown represents the mean ± SEM of n ≥ 3.

**Growth analysis**

Growth analysis by a spotting test (Kwolek-Mirek and Zadrag-Tecza 2014) was performed using 1 × 10^6 cell/ml aliquots (determined by direct cell count of culture dilutions) taken from cultures at OD_600 between 0.5-0.8. Strains bearing deletion mutations of the genes encoding bait proteins, their corresponding newly identified protein partners, and double-mutant strains were compared with wild type strain BY4742 controls (Table S1). For agar plate assays, 3 μl drops taken from 1/10 serial dilutions of the working culture (ranging from 10^8 to 10^2 cell/ml) were inoculated in triplicate on CSM agar plates under the following conditions: normal conditions (30°C, no treatment), oxidative stress (1 mM H_2O_2), cell wall stress (75 ng/ml CS) and plasma membrane stress (100 mM FCZ). Plates were incubated at 30°C for 2-3 days. Relative growth on agar plates was quantified by comparing the density of growth within each drop displayed by the wild type strain across all dilutions, to the density of growth displayed by the mutant strains across the same dilution series. Relative growth measurements were classified as follows: density of growth equivalent to a wild type strain was described as “No Phenotype” (NP), greater than the wild type by one or more serial dilutions was described as “Resistant” (R), less than the wild type by one or more serial dilutions was described as “Sensitive” (S) (Figures 4A-4C and Tables S3-S6). Strains that exhibited the (S) phenotype in the spotting tests were subjected to corroboration by the Colony Forming Units (CFU) assay (Table S7) (Kwolek-Mirek and Zadrag-Tecza 2014).

**Immunoprecipitation coupled with Mass Spectrometry (IP-MS)**

Five (5) ml cultures containing Wsc2p-GFP, Wsc3p-GFP and BY4742 strains were grown in YPD at 30°C overnight. 25 ml yeast cultures in YPD media were inoculated with these overnight cultures and grown strains were grown in YPD at 30°C (Table S7) (Kwolek-Mirek and Zadrag-Tecza 2014).

**Bioinformatics analysis**

Protein sequences with homology to Wsc2p and Wsc3p and the 12 iMYTH interactors that could be retrieved by conducting a comprehensive homology search in the public biological databases from The UniProt Consortium (2017) and European Molecular Biology Laboratory - European Bioinformatics Institute (EMBL-EBI) with Multiple Sequence Alignment Programs were obtained by using specific Structure Query Language (SQL) queries (Nicholas et al. 2000). Bioinformatics analysis was performed using the application Multiple Sequence Comparison by Log-Expectation (MUSCLE) (Edgar 2004) (Table 3). The acquired data were evaluated using two criteria: The first criterion was selection of the proteins that were conserved in yeast Saccharomyces cerevisiae, Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans, and Homo sapiens. This selection was performed by a series of specific queries in SQL using the following names and alternative names for all proteins excluding Gt1lp, Zeo1p, and Wsc1p that were not conserved in H. sapiens. The first criterion was met by proteins: Bmh1p, Cpr1p, Egd1p, Egd2p, Mps1p, Ras2p, Tma7p, Yck1p, Yck2p, Ypl199cp, Mtl2, Mtl1p, Wsc2p, and Wsc3p. The second criterion was inclusion of all proteins (interactors and mechanosensory proteins) that shared homology with Saccharomyces cerevisiae and at least one of the following fungi: Aspergillus fumigatus, Candida albicans, or Cryptococcus neoformans, and did not share any homology with Homo sapiens.

**Data availability**

All strains and reagents are available upon request to the corresponding author. Supplemental Materials: Table S1. Double mutant strains used in this study; Table S2. Primers used in this study; Table S3. Susceptibility profiles of single mutant strains of Wsc2p interactors; Table S4. Susceptibility profiles of double mutant strains of Wsc2p and interactors; Table S5. Susceptibility profiles of single mutant strains of Wsc3p interactors; Table S6. Susceptibility profiles of double mutant strains of Wsc3p and interactors; Table S7. Colony Forming Units (CFU) assay of hypersensitive mutant strains of Wsc2p, Wsc3p, and their interactors; Figure S1. A representative spotting test for growth of null mutant strains of Wsc2p, Wsc3p, and their interactors treated with 75 ng/ml CS; Figure S2. Western blot analysis of
Table 1). Unique interactors were verified and cDNAs sequenced. Fifteen out of 58 different proteins identified and cDNAs sequenced. Fifteen out of 58 different proteins identified in selection media, and the corresponding plasmids were shared by the bait strains. With the exception of Zeo1p, these interactors were novel and not reported previously in the Saccharomyces Genome Database (yeastgenome.org) (Cherry et al., 2012). A map of the interaction network for the 12 interactors of Wsc2p and Wsc3p identified by iMYTH and confirmed by BDT was generated (Figure 3). The SGD reports 20 additional physical interactors for Wsc2p and 13 for Wsc3p, identified by other methods, that were not identified in our iMYTH screen. The biological functions described in the SGD (yeastgenome.org) (Cherry et al., 2012) for Wsc2p, Wsc3p, and their iMYTH protein interactors are listed in Table 1.

Assessment of Wsc2p and Wsc3p interactor function in resistance to antifungal treatments

We next evaluated the growth of yeast bearing single or double deletions of the genes defined by the iMYTH interactions. Assays were performed in the absence of drug and in the presence of the antifungals caspofungin (CS), an inhibitor of the fungal β-1,3-glucan synthetase, an essential component of the yeast cell wall, thereby disturbing the integrity of the fungal cell wall (Deresinski and Stevens 2003) and fluconazole (FCZ), which inhibits fungal ergosterol synthesis by inhibiting a P-450 enzyme (C-14 α-demethylase) required for the formation of ergosterol, an essential component of the fungal cytoplasmic membrane (Roemer and Krysan 2014). Drug sensitivity was defined as described in the Materials and Methods (Figures 4A and 4B).

To a first approximation, with the exception of the slow growing ypl199cΔ mutant, all single gene deletion strains grew as well as the BY4742 wildtype strain in the absence of either FCZ (Figure 4A, 4B) or CS (Figure S1). The wsc2Δ strain exhibited a sensitive phenotype (S) with FCZ treatment (Figure 4A) and a normal growth phenotype (NP) upon treatment with CS (Figure S1). Strains msa1Δ, gti1Δ and bnh1Δ displayed a sensitive phenotype (S) when treated with FCZ (Figure 4A, Figure 4B, Table S3, and Table S5 respectively) and a normal phenotype (NP) in untreated and CS treated cultures (Figure S1, Table S3, and Table S5). The FCZ sensitivity (S) phenotypes of the single gene deletion strains were all validated by a Colony Forming Unit (CFU) assay (Table S7) (Kwolek-Mirek and Zadrag-Tecza 2014). In contrast, ypl199cΔ cultures displayed slow growth (S') in the untreated and CS treated cultures, and a normal growth phenotype (NP) in the FCZ treated culture (Figure 4A, Table S3). Thus, the slow growth of the ypl199cΔ mutant was attributed to the YPL199c gene deletion with no effect attributed to the stress treatments.

Double mutant strains with preyΔsensorΔ combinations were evaluated to determine if any combination displayed a synthetic mutant phenotype. PreyΔwsc2Δ double mutants that grew similarly to the single strongest mutant phenotype were classified as PPIs that likely participate in a common stress response pathway. Mutant combinations of preyΔwsc2Δ that displayed growth phenotypes that were different from their respective single mutant strains were classified as PPIs potentially acting in different stress response pathways. In the absence of added drug, the egd2Δwsc2Δ, msa1Δwsc2Δ, yck2Δwsc2Δ, and egd2Δwsc3Δ double mutants all showed more severe growth inhibition than the corresponding single mutants (Figure 4A, 4B). Inexplicably, the slow growth of the ypl199cΔ strain was suppressed in the ypl199cΔwsc2Δ double mutant background.

In the presence of FCZ, the gti1Δwsc2Δ strain exhibited a sensitive phenotype (Figure 4A, Table S4) as did the individual wsc2Δ and gti1Δ strains (Figure 4A, Table S3, Table S7). The yck2Δwsc2Δ strain exhibited an FCZ-resistant phenotype (R) (Figure 4A, Table S4) while the individual yck2Δ and wsc2Δ strains displayed (NP) and (S) phenotypes, respectively (Figure 4A, Table S3). As noted above, the

RESULTS

Confirmation of expression and localization of Wsc2p and Wsc3p iMYTH bait constructs

An iMYTH assay was conducted to detect protein-protein interactions of the mechanosensors Wsc2p and Wsc3p. Wsc2p and Wsc3p prey proteins were used to identify cytoplasmic and membrane-associated prey proteins in vivo as described previously (Snider et al., 2010; Santiago-Cartagena et al. 2019). Fluorescence microscopy (Figure 1) was used to demonstrate that both the Wsc2p and Wsc3p bait proteins were expressed and correctly localized to the plasma membrane. The clustered appearance of the Wsc2p-YFP and Wsc3p-YFP fluorescence at the cell periphery was reminiscent of the pattern previously observed for Mid2p-YFP (Santiago-Cartagena et al. 2019). The Wsc2p and Wsc3p bait proteins did not self-activate in the absence of interacting prey using the NAD/NADG control tests (Figure 2) (Snider et al. 2010).

Protein interaction network map of Wsc2p and Wsc3p

Prey proteins that interacted with the Wsc2p and Wsc3p baits were identified in selection media, and the corresponding plasmids recovered and cDNAs sequenced. Fifteen out of 58 different proteins identified (26%) were confirmed by Bait Dependency Tests (BDTs) (Snider et al. 2010) (Figure 2). These encoded 10 protein interactors for Wsc2p (Egd1p, Egd2p, Gtt1p, Msa1p, Ras2p, Tma7p, Yck1p, Yck2p, Ypl199c, Zeo1p) and 5 protein interactors for Wsc3p (Bnh1p, Cpr1p, Egd2p, Yck1p, and Zeo1p) (Figure 2 and Table 1) of which Yck1p, Zeo1p, and Egd2p were shared by Wsc2p and Wsc3p (Figure 3 and Table 1). Unique interactors were verified by cross-testing in each of

Figure 1 Representative DIC and YFP images for the localization of Wsc2p and Wsc3p in the plasma membrane. A) and C) display DIC images for Wsc2p L40 L3 and Wsc3p L40 L3 strains respectively. B) and D) show fluorescent images of Wsc2p L40 L3 and Wsc3p L40 L3 strains respectively. Images were acquired with a Leica DMI 6000B Inverted Confocal Microscope with a field of view of 16 μm. YFP = Yellow Fluorescent Protein, DIC = Differential Interference Contrast.

phospho-Slt2p (P-Slt2p) levels in null mutant strains of Wsc2p and Yck2p treated with 75 ng/ml CS. Supplemental material available at figshare: https://doi.org/10.25387/g3.12453707.
egd2Δwsc2Δ, msa1Δwsc2Δ, yck2Δwsc2Δ, and egd2Δwsc3Δ double mutants all showed some growth inhibition in the absence of FCZ yet each double mutant grew at least as well as the BY4742 control when FCZ was present (Figure 4A, Figure 4B, Table S4, and Table S6 respectively).

Strains bearing wscΔ or bmh1Δ single mutations were sensitive (S) to FCZ but no double mutant combinations of preyΔwscΔ strains showed sensitivity to FCZ or CS (Figure 4A, Figure 4B, Table S5, and Table S6). Curiously, the double mutant strain egd1Δwsc2Δ exhibited normal growth (NP) under all conditions tested. At the same time, the egd2Δwsc2Δ and egd2Δwsc3Δ displayed diminished growth in untreated cultures, and resistant growth (R) in response to FCZ treatment (Figures 4A, Figure 4B, Table S4, and Table S6 respectively).

Assessment of Wsc2p and Wsc3p interactor function in activation of the CWI pathway

We wished to determine if the PPIs that acquired a sensitive growth phenotype (S) when mutated were also needed to activate the CWI pathway. To test this, mutant strains containing deletions of genes encoding Wsc2p or Wsc3p (sensorΔ), their interactors (preyΔ), or double mutant combinations (preyΔsensorΔ) that exhibited a growth defect in the previous analysis were assayed for the accumulation of hyper-phosphorylated Slt2p (P-Slt2p), the readout for CWI pathway activation. Treatments with antifungal agents CS (Figure S2), FCZ (Figure 5A and 5B), or H2O2 (Figure 5C) were as described in the previous section. PPIs that exhibited the (S) or (R) growth phenotypes with stress treatment were scored for CWI pathway activation. Strains bearing single mutant yck2Δ and yck2Δwsc2Δ (Figure 5A), and gtt1Δ and gtt1Δwsc2Δ (Figure 5B), failed to activate the CWI pathway with FCZ addition. Taken together with the results presented above, there...
CWI activation and growth phenotype under FCZ stress conditions does not appear to be a simple consistent correlation between enzymes involved in the cellular response to oxidative stress, were proteins (Santiago-Cartagena et al. 2019). We find that Gtt1p a dual function enzyme with glutathione S-transferase and peroxidase activity interacts with Wsc2p. Similar to the wildtype, mutant strains bearing wsc2Δ, gtt1Δ, and gtt1Δwsc2Δ displayed normal growth phenotypes (NP) (Figure 4C, Table S3, Table S4) and did not activate the CWI pathway significantly upon treatment with 1mM H2O2 (Figure 4C). In contrast, the ras2Δ and ras2Δwsc2Δ positive control strains showed the expected sensitive (S) growth in response to H2O2 treatment (Figure 4C, Table S3 and Table S4) with elevated basal levels of P-Slt2p and a diminished capacity for CWI pathway.

Table 1 Wsc2p and Wsc3p interacting proteins identified by iMYTH assay and confirmed by BDT

| Name  | Systemic Name | Bait Gene | Description (According to SGD, http://www.yeastgenome.org) |
|-------|---------------|-----------|------------------------------------------------------------|
| BMH1  | YER177W       | WSC3P     | 14-3-3 protein, major isoform; controls proteome at post-transcriptional level, binds proteins and DNA, involved in regulation of exocytosis, vesicle transport, Ras/MAPK and rapamycin-sensitive signaling, aggresome formation, spindle position checkpoint; protein increases in abundance and relative distribution to the nucleus increases upon DNA replication stress; antia apoptotic gene similar to human 14-3-3; BMH1 has a paralog, BMH2, that arise from whole genome duplication. |
| CPR1  | YDR155C       | WSC3P     | Cytoplasmic peptidyl-prolyl cis-trans isomerase (cyclophilin); catalyzes the cis-trans isomerization of peptide bonds N-terminal to proline residues; binds the drug cyclosporin A; N-terminally propionylated in vivo; protein abundance increases in response to DNA replication stress. |
| EGD1  | YPL037C       | WSC2P     | Subunit beta1 of the nascent polypeptide-associated complex (NAC); involved in protein targeting, associated with cytoplasmic ribosomes; enhances DNA binding of the Gal4p activator; homolog of human BTF3b; EGD1 has a paralog, BTT1, that arise from the whole genome duplication. |
| EGD2  | YHR193C       | WSC2P, WSC3P | Alpha subunit of the nascent polypeptide-associated complex (NAC); involved in protein sorting and translocation; associated with cytoplasmic ribosomes. |
| GTT1  | YIR038C       | WSC2P     | ER associated glutathione S-transferase; capable of homodimerization; glutathione transferase for Yvc1p vacuolar cation channel; expression induced during the diauxic shift and throughout stationary phase; functional overlap with Gtt2p, Gnx1p, and Gnx2p. |
| MSA1  | YOR066W       | WSC2P     | Activator of G1-specific transcription factors MBF and SBF; involved in regulation of the timing of G1-specific gene transcription and cell cycle initiation; localization is cell-cycle dependent and regulated by Cdc28p phosphorylation; MSA1 has a paralog, MSA2, that arise from the whole genome duplication. |
| RAS2  | YNL098C       | WSC2P     | GTP-binding protein; regulates nitrogen starvation response, sporulation, and filamentous growth; farnesylation and palmitoylation required for activity and localization to plasma membrane; homolog of mammalian Ras proto-oncogenes; RAS2 has a paralog, RAS1, that arise from the whole genome duplication. |
| TMA7  | YLR262C-A     | WSC2P     | Protein of unknown that associates with ribosomes; null mutant exhibits translation defects, altered polyribosome profiles, and resistance to the translation inhibitor anisomycin; protein abundance increases in response to DNA replication stress. |
| YCK1  | YHR135C       | WSC2P, WSC3P | Palmitoylated plasma membrane-bound casein kinase I (CK1) isoform; shares redundant functions with Yck2p in morphogenesis, proper septin assembly, endocytic trafficking, and glucose sensing; stabilized by Sod1p binding in the presence of glucose and oxygen, causing glucose repression of respiratory metabolism; involved in the phosphorylation and regulation of glucose sensor Rgt2p; YCK1 has a paralog, YCK2, that arise from the whole genome duplication. |
| YCK2  | YNL154C       | WSC2P     | Palmitoylated plasma membrane-bound casein kinase I (CK1) isoform; shares redundant functions with Yck1p in morphogenesis, proper septin assembly, endocytic trafficking, and glucose sensing; stabilized by Sod1p binding in the presence of glucose and oxygen, causing glucose repression of respiratory metabolism; involved in the phosphorylation and regulation of glucose sensor Rgt2p; YCK2 has a paralog, YCK1, that arise from the whole genome duplication. |
| YPL199C | YPL199C | WSC2P | Putative protein of unknown function; predicted to be palmitoylated. |
| ZEO1  | YOL109W       | WSC2P, WSC3P | Peripheral membrane protein of the plasma membrane; interacts with Mid2p; regulates the cell integrity pathway mediated by Pkc1p and Slt2p; the authentic protein is detected in a phosphorylated state in highly purified mitochondria. |
Re-validation of iMYTH protein-protein interactions by Immunoprecipitation-Mass Spectrometry (IP-MS)

To re-validate the results of previous iMYTH BDT confirmations with Wsc2p and Wsc3p, we performed immunoprecipitation (IP) of endogenously expressed GFP-tagged Wsc2p and Wsc3p from total protein extracts and coupled the IPs with Mass Spectrometry (IP-MS) analysis as described previously (Santiago-Cartagena et al. 2019). By this method, the iMYTH interactors were re-validated as interactors of Wsc2p while Bmh1p, Egd2p, Yck1p, and Ras2p were re-validated as interactors of Wsc3p, both of which were previously reported in the SGD PPI database. We have stated previously that protein interactors re-validated by IP-MS are likely to represent proteins with the strongest interactions (Santiago-Cartagena et al. 2019). In contrast, iMYTH protein interactors confirmed by the bait dependency test that were not identified in the IP-MS assay may represent transient associations or protein interactions too weak to survive the extraction process. However, we recognize that the introduction of protein tags and other experimental variables may also impact protein recovery. An integrative interactome map with the re-validated IP-MS interactors of Wsc2p and Wsc3p, as well as our previous data from studies with re-validated interactors of Wsc1p and Mid2p, their corresponding biological functions, and cellular compartment information is provided (Figure 6).

Bioinformatics analysis for identification of homologous proteins in other fungal species

Bioinformatics analysis was performed using the application MUSCLE (Edgar R.C., 2004) to identify protein sequences with homology to Wsc2p and Wsc3p and the 12 iMYTH interactors. Homologous proteins for Wsc2p, Wsc3p and their interactors were identified in Aspergillus fumigatus, Candida albicans, Cryptococcus neoformans, and Homo sapiens (Table 3). Homologous sequences for all except Gtt1p, Zeo1p, and Wsc1p were identified in humans making this fungal specific set of proteins potentially valuable therapeutic targets.

DISCUSSION

This study describes 12 novel protein-protein interactions (PPIs) identified by iMYTH screening, collectively referred to as “interactors” of the mechanosensors Wsc2p and Wsc3p of S. cerevisiae. With the exception of Zeo1p, all the iMYTH interactors of Wsc2p and Wsc3p that we identified were novel. A protein interaction network or “interactome” was assembled using the validated PPIs identified by iMYTH (Figure 3). A second interactome image presents the seven iMYTH interactors of Wsc3p that were re-validated by IP-MS analysis combined with previously defined iMYTH interactors of Wsc1p and Mid2p (Figure 6) (Santiago-Cartagena et al. 2019). This display reveals multiple redundant PPIs among these mechanosensory proteins. Interestingly, among the iMYTH interactors of Wsc2p and Wsc3p that were re-validated by IP-MS, only Bmh1p exhibited a growth defect in functional tests with FCZ. The interactors Gtt1p and Msa1p which also exhibited growth defects with FCZ were not re-validated by IP-MS. Therefore, we suggest that although re-validation of iMYTH results by alternative methods such as IP-MS provides important information about the nature of these PPIs, this criterion alone should not be used to exclude PPIs of potential interest.

Expression of Wsc2p appeared to be more relevant for stress resistance than Wsc3p if judged by the sensitive responses of three of its unique interactors (Ras2p, Yck2p, and Gtt1p) in functional tests with the potent antifungal drug FCZ and the strong oxidant H2O2. Specifically, gtt1Δ and wsc2Δ in both the single or double mutant.
strains on CSM, or treated with 1 mM hydrogen peroxide (H2O2). The treatment (CSM), treated with 2% DMSO vehicle, or treated with 100 μM Fluconazole (FCZ); B: wsc3Δ interactors on CSM, treated with 2% DMSO vehicle, or treated with 100 μM FCZ; and C: gtt1Δ and ras2Δ strains on CSM, or treated with 1 mM hydrogen peroxide (H2O2). The plates were inspected after two days of incubation. Three replicates were done for each experiment.

Figure 4 A representative spotting test of mutant strains of Wsc2p and Wsc3p and their interactors exposed to stress conditions. Identical volumes representing 10-fold serial dilutions (10^8-10^2) of wild type (BY4742), single, and double mutants, were spotted onto CSM plates and incubated at 30°C. From left to right, A: wsc2Δ interactors with no treatment (CSM), treated with 2% DMSO vehicle, or treated with 100 μM Fluconazole (FCZ); B: wsc3Δ interactors on CSM, treated with 2% DMSO vehicle, or treated with 100 μM FCZ; and C: gtt1Δ and ras2Δ strains on CSM, or treated with 1 mM hydrogen peroxide (H2O2). The plates were inspected after two days of incubation. Three replicates were done for each experiment.

These results suggest that Gtt1p and Wsc2p have an additive effect to promote resistance to FCZ treatment. Yeast bearing egd2Δ or yck2Δ in combination with wsc2Δ, exhibited a resistant growth phenotype (R) with FCZ treatment. The egd2Δwsc3Δ double mutant responded similarly. These results indicate that both Egd2p, Yck2p down-regulate growth when membrane stress is induced by FCZ treatment. Deletion of EGD2 and YCK2 in the wsc2Δ background therefore reverses the sensitivity to FCZ in wsc2Δ and wsc3Δ mutants. We speculate that the absence of Egd2p in the nascent polypeptide associated complex (NAC), which results in diminished translational capacity (Kirstein-Miles et al. 2013), enhances fitness in the wsc2Δ background when membrane biogenesis is impaired by FCZ. What links the control of translational capacity to drug resistance is not known. A possible role for Yck2p as the phosphorylating kinase of Wsc2p, proposed to be a negative regulatory step in CWI activation, is discussed below.

The Gtt1p interactor of Wsc2p is a plasma membrane localized enzyme with bifunctional glutathione S-transferase and peroxidase activities. Conceivably, the loss of Gtt1p activity during H2O2 treatment might impair growth through oxidative damage to important cellular proteins (Hawkins and Davies 2019). However, the absence of either Gtt1p and/or Wsc2p had little or no impact on growth or CWI pathway activation in response to H2O2 treatment, although both proved important for efficient growth in response to FCZ treatment implicating them in a specific role for FCZ resistance.

Based on the prevailing model, the phosphorylation of serine residues within the regulatory C-terminal region of the Wsc1 mechanosensory protein inhibits Rom2p binding (Vay et al. 2004). The shared Yck1p and Yck2p interactors are serine-threonine protein kinases. These protein kinases may phosphorylate this serine-rich motif, specifically S319, S320, S322, S323, at the Wsc1p C-terminus (Vay et al. 2004) and equivalent positions within the Wsc2p, Wsc3p and Mid2p mechanosensory proteins that likewise interact with Yck1p or Yck2p. In such a case, the absence of these phosphorylation events would be predicted to enhance CWI activation (viewed as elevated P-Slt2p) under stress conditions. While the yck2Δ strain displayed a trend toward elevated basal levels of P-Slt2p in both the FCZ treated and untreated cultures, which is consistent with this prediction (Figure 5A), this strain exhibited slow growth (S) under FCZ treatment (Figure 4A). In contrast, the yck2Δwsc2Δ strain displayed suppressed levels of CWI activation (Figure 5A) yet this strain exhibited resistant growth (R) under FCZ treatment (Figure 4A). These observations indicate that independent of its putative negative regulatory role in CWI activation, Yck2p expression is required for normal growth under FCZ treatment conditions (exhibited by the slow growth of a yck2Δ strain, Figure 4A), while it is dispensable under the same conditions when Wsc2p is absent (exhibited by the resistant growth of a yck2Δwsc2Δ strain, Figure 4A). We propose that in a BY4742 control strain under normal culture conditions, Yck2p is capable of suppressing Wsc2p interaction with Rom2p through phosphorylation of the Wsc2p C-terminus (maintaining normal growth and preventing premature CWI activation). Under FCZ treatment conditions, Wsc2p may be released from this suppression state either by a deletion of Yck2p in yck2Δ strains, or by a proposed enzymatic dephosphorylation of its C-terminus (maintaining normal growth equivalent to BY4742 and elevated CWI activation). The absence of Yck2p in the yck2Δ mutant therefore elevates the baseline level of P-Slt2p, a phenotype that resembles elevated steady state CWI activation in ras2Δ mutants (Park et al. 2005, Santiago-Cartagena et al. 2019). The absence of both Wsc2p and Yck2p in a double mutant yck2Δwsc2Δ
Figure 5 Western blot analysis of phospho-Slt2p (P-Slt2p) levels in mutant strains of Wsc2p and Wsc3p and their interactors treated with (A), (B): 100 μM FCZ, and (C) 1 mM H2O2. Wild type, single, and double mutant cultures were treated with each chemical for 1 hr at 27°C. Western blots of total protein extracts derived from treated and control cultures were incubated with anti-phospho p44/42 MAPK (P-Slt2p) and anti-phosphoglycerate kinase (Pgk1p) antibodies as the loading control.

The Hek2p RNA binding protein was previously identified by Hasegawa et al. (2008) as a physical interactor of Wsc2p as illustrated in Figure 6. Interestingly, Hek2p is reported to represses translation of ASH1 mRNA while Yck1p-dependent phosphorylation of the RNA-binding protein Khdlp activates ASH1 mRNA translation. While Khdlp is not an interactor of Wsc2p, the PPIs of Yck1p and Hek2p with Wsc2p raises the possibility that their association with Wsc2p at the plasma membrane may be necessary for regulating ASH1 mRNA translation in daughter cells.

Ras2p is a small GTPase that regulates adenylate cyclase activity and cAMP synthesis (Conrad et al. 2014). The yeast Ras2p shares approximately 65% of amino acid sequence homology with the conserved human KRAS, HRAS, and RRAS proteins (Table 3). An interaction reported previously between ras2Δ and rom2Δ mutants in yeast suggested that Ras2p expression modulates Rom2p-dependent activation of the CWI pathway (Park et al. 2005). In the same study, while the ras2Δ mutant accumulated elevated baseline levels of phospho-Slt2p (corroborated in our studies), it down regulated MAPK activation in response to heat shock. Overexpression of Rom2p did not overcome the down regulation of CWI in this ras2Δ mutant. In our current study, Ras2p was confirmed as an interacting partner of Wsc2p and shown to have a pivotal role in response to oxidative stress induced by H2O2, consistent with other reports (Vilella et al. 2005). We find that ras2Δ mutants exhibited elevated baseline levels of phospho-Slt2p in untreated cells and fail to accumulate additional phospho-Slt2p following oxidative stress treatment. An explanation for how the P-Slt2p levels drop in the ras2Δ mutants during H2O2 treatment, rather than remaining stable at the untreated baseline levels, may be that H2O2 shuts down the CWI pathway almost completely in these strains. Normal turnover of the protein would then produce a drop in P-Slt2p steady state levels to below untreated control levels.

The physical interaction of Ras2p with Wsc2p and Wsc3p reported here supports the hypothesis that Ras2p may modulate binding of Rom2p at the C-terminus of multiple Wsc-family mechanosensory proteins simultaneously. Going forward, it will be important to determine if Ras2p and Rom2p directly bind to the C-terminal sequence of Wsc2p (and perhaps other mechanosensors) or interact via common linker proteins.

Multiple strains such as ypl199cΔ, msa1Δwsc2Δ, yck2Δwsc2Δ, and egd2Δwsc3Δ (Figure 4A and 4B, Tables S3, S4, and S6) exhibited synthetic growth defects under control conditions that were not described in the Materials and Methods. Numerical values shown within each histogram represent fold-change of the P-Slt2p intensity in each sample adjusted to the P-Slt2p intensity of wild type control (WT) each normalized against Pgk1p. Each data point shown represents the mean ± SEM of n ≥ 3.
changed by the addition of the CS or FCZ chemical stressors. While the underlying basis of these genetic interactions is unknown, presumably they relate to the functions of the encoded proteins outside the context of CWI pathway response. The *YPL199c* interactor encodes a protein of unknown function that is predicted to be palmitoylated (Ren et al. 2008). This study contributes a novel descriptor for the

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**Table 2 Peptides counts and probability percentages for Wsc2p and Wsc3p interactors identified by iMYTH and validated by GFP immunoprecipitation coupled to Mass Spectrometry analysis (IP-MS)**

| Protein Name | WT^a | Wsc2^a | Wsc3^a | Wsc3^b |
|--------------|------|--------|--------|--------|
|              | TP   | TP     | TP     | TP     | TP     | TP   | TP     |
| Wsc2         | 156(99.58) | 87(99.58) | 12(99.58) | 0   | 0   | 0   |
| Wsc3         | 0   | 0 | 0 | 0 | 0 | 0 |
| Bmh1         | 0   | 0 | 0 | 0 | 0 | 0 |
| Egd2         | 4(99.52) | 2(99.52) | 2(96.06) | 3(99.58) | 3(99.45) | 0   |
| Yck2         | 3(99.58) | 2(99.58) | 2(99.58) | 2(99.58) | 2(99.58) | 0   |
| Hek2         | 2(99.58) | 3(99.58) | 0 | 0 | 0 |
| Tcb3         | 6(99.58) | 2(99.58) | 2(99.58) | 2(99.58) | 1(97.33) | 1(99.58) |
| Yck1         | 3(99.58) | 2(99.58) | 4(99.58) | 1(99.58) | 3(99.58) | 5(99.58) |
| Yck2         | 2(99.58) | 2(99.58) | 2(99.58) | 2(99.58) | 7(99.58) |
| Ras2^b       | 6(99.58) | 0 | 0 | 0 | 2(99.58) |

^aTP = Total peptides, (Probability percentage), N = 3.

^bTP = Total peptides, (Probability percentage), N = 1.

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**Figure 6** Interactome of *Wsc1p*, *Wsc2p*, *Wsc3p*, and *Mid2p* interactor proteins validated by BDT, AP-WB, and/or IP-MS. Geometric shapes of the nodes represent the cellular localization of the proteins. *Wsc1p*, *Wsc2p*, *Wsc3p*, and *Mid2p* are the bait proteins and the surrounding nodes represent prey proteins. Node fill color-code indicates the biological process for each protein. All edges indicate a physical protein-protein interaction.
| Interactor or Sensor Protein Name | Yeast (Query Sequence) | Aspergillus fumigatus | Candida albicans | Cryptococcus neoformans | Homo sapiens |
|----------------------------------|------------------------|-----------------------|------------------|------------------------|-------------|
| Bmh1                             | BMH1                   | 2                     | 2                | 3                      | 2           |
|                                  | Y699_03118             | BMH1                  | BMH1             | CNBI2830               | YWHAE       |
|                                  | Y699_03834             |                       |                  | CNAG_05235             | HEL2        |
|                                  |                        |                       |                  | CNL03930               |             |
| Cpr1                             | CPR1                   | 3                     | 2                | 3                      | 3           |
|                                  | Y699_01807             | BMH1                  | BMH1             | CPA1                   |             |
|                                  | asp f 27               |                       |                  | CPA2                   |             |
|                                  | Y699_06153             |                       |                  | CPA2                   |             |
|                                  |                        |                       |                  | N/A                    |             |
| Egd1                             | EGD1                   | 1                     | 2                | 3                      | 3           |
|                                  | Y699_07424             | BMH1                  | BMH1             | CNAG_03627             |             |
|                                  |                        |                       |                  | CPA1                   |             |
|                                  |                        |                       |                  | CPA2                   |             |
|                                  |                        |                       |                  | N/A                    |             |
| Egd2                             | EGD2                   | 0                     | 2                | 3                      | 3           |
|                                  |                        |                       |                  | BMH1                   |             |
|                                  |                        |                       |                  | BMH1                   |             |
|                                  |                        |                       |                  | N/A                    |             |
| Gtt1                             | GTT1                   | 3                     | 4                | 0                      | 0           |
|                                  | Y699_09395             | BMH1                  | BMH1             | CNAG_03621             |             |
|                                  | Y699_02150             |                       |                  | CNAG_03621             |             |
|                                  | Y699_05729             |                       |                  | CNAG_03621             |             |
|                                  |                        |                       |                  | CNAG_03621             |             |
| Msa1                             | MSA1                   | 0                     | 0                | 0                      | 2           |
|                                  |                        |                       |                  | ZNF384                 |             |
|                                  |                        |                       |                  | ZNF384                 |             |
| Ras2                             | RAS2                   | 13                    | 8                | 8                      |             |
|                                  |                        |                       |                  | RAS1                   |             |
|                                  |                        |                       |                  | CNAG_04119             |             |
|                                  |                        |                       |                  | CNBH4090               |             |
|                                  |                        |                       |                  | CNJ04280               |             |
|                                  |                        |                       |                  | RAS1                   |             |
|                                  |                        |                       |                  | CNAG_00293             |             |
|                                  |                        |                       |                  | NRAS                   |             |
|                                  |                        |                       |                  | HRAS                   |             |
| Yck1                             | YCK1                   | 1 Y699_03846          | 3                | 5                      |             |
|                                  |                        |                       |                  | CNBAS210               |             |
|                                  |                        |                       |                  | CNBAS210               |             |
|                                  |                        |                       |                  | CNAG_00556             |             |
|                                  |                        |                       |                  | CNAG_00556             |             |

(continued)
| Organisms | Yeast (Query Sequence) | Aspergillus fumigatus | Candida albicans | Cryptococcus neoformans | Homo sapiens |
|-----------|-----------------------|----------------------|-----------------|------------------------|--------------|
| Yck2      | YCK2                  | 1                    | 2               | 6                      | 5            |
|           |                       |                      | CAALFM_C208270CA YCK2 | CNBA5210              | CSNK1G3      |
|           |                       |                      |                 | CNBA5210              | CSNK1G3      |
|           |                       |                      |                 | CNAG_00556             | CSNK1G3      |
|           |                       |                      |                 | CNA05390              | CSNK1G2      |
|           |                       |                      |                 | CNA05390              | CSNK1G3      |
|           |                       |                      |                 | CNAG_00556             |              |
| Ypl199c   | YPL199C               | 1                    | 1               | 3                      | 3            |
|           |                       |                      | Y699_05052      | CNBF2620              | N4BP2        |
|           |                       |                      |                 | CNFO2090              | N4BP2        |
|           |                       |                      |                 | CNAG_05769             | N4BP2        |
| Zeo1      | ZEO1                  | 0                    | 1               | 0                      | 0            |
|           |                       |                      | CTA2            | CNAG_05550             |              |
|           |                       |                      |                 | CNH01980              |              |
|           |                       |                      |                 | CNH01980              |              |
|           |                       |                      |                 | CNH01980              |              |
| Mid2      | MID2                  | 0                    | 2               | 6                      | 2            |
|           |                       |                      | orf19.4906      | C365_06939             | TPRXL        |
|           |                       |                      |                 | CNAG_05550             |              |
|           |                       |                      |                 | CNAG_05550             |              |
|           |                       |                      |                 | CND03720              |              |
|           |                       |                      |                 | CNH01980              |              |
|           |                       |                      |                 | CNH01980              |              |
|           |                       |                      |                 | CNH01980              |              |
| Mtl1      | MTL1                  | 0                    | 3               | 8                      | 8            |
|           |                       |                      | orf19.4906      | CNBG2510              | MUC5AC       |
|           |                       |                      |                 | CNBG2510              | MUC21        |
|           |                       |                      |                 | CNBG2510              | MUC21        |
|           |                       |                      |                 | CNAG_00475             | MUC21        |
|           |                       |                      |                 | CNAG_00475             | MUC21        |
|           |                       |                      |                 | CNAG_00475             | FLJ50027     |
| Wsc1      | SLG1                  | 1                    | 13              | 13                     | 0            |
|           |                       |                      | CENPK1137D_2030 | CNAG_05550             |              |
|           |                       |                      |                 | CNH01980              |              |
|           |                       |                      |                 | CNH01980              |              |
|           |                       |                      |                 | CNBL1980              |              |
|           |                       |                      |                 | CNAG_00668             |              |
|           |                       |                      |                 | CND03720              |              |
|           |                       |                      |                 | CNH01980              |              |
|           |                       |                      |                 | CNH01980              |              |
|           |                       |                      |                 | CNH01980              |              |
| Wsc2      | WSC2                  | 1                    | 5               | 8                      | 3            |
|           |                       |                      | Y699_05984      | CNAG_07659             | MUC5AC       |
|           |                       |                      |                 | CNH01980              | TPRXL        |
|           |                       |                      |                 | CNBL1980              |              |
|           |                       |                      |                 | CNAG_05550             |              |
|           |                       |                      |                 | CNAG_03328             |              |
|           |                       |                      |                 | CNBG2260              |              |
|           |                       |                      |                 | CNH01980              |              |
|           |                       |                      |                 | CNH01980              |              |
|           |                       |                      |                 | CNH01980              |              |
| Wsc3      | WSC3                  | 1                    | 4               | 3                      | 3            |
|           |                       |                      | Y699_09176      | CNAG_05550             | MUC5AC       |
|           |                       |                      |                 | CNH01980              | TPRXL        |
|           |                       |                      |                 | CNBL1980              |              |
|           |                       |                      |                 | CNAG_05550             |              |
|           |                       |                      |                 | CNAG_05550             |              |
|           |                       |                      |                 | CNAG_05550             |              |
|           |                       |                      |                 | CNAG_05550             |              |
|           |                       |                      |                 | CNAG_05550             |              |
Ypl199c protein as a physical interactor of Wsc2p, consistent with its proposed membrane association. Bmh1p was the only interactor of Wsc3p other that Wsc3p itself that exhibited sensitivity to FCZ treatment (Figure 4B, Table S5). Bmh1p is described as a 14-3-3 protein that has been linked to Ras/MAPK and rapamycin-sensitive treatment (Figure 4B, Table S5).

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