A Novel Function for Hog1 Stress-Activated Protein Kinase in Controlling White-Opaque Switching and Mating in Candida albicans

Shen-Huan Liang, Jen-Hua Cheng, Fu-Sheng Deng, Pei-An Tsai, Ching-Hsuan Lin
Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, Taipei, Taiwan

Candida albicans is a commensal in healthy people but has the potential to become an opportunistic pathogen and is responsible for half of all clinical infections in immunocompromised patients. Central to understanding C. albicans behavior is the white-opaque phenotypic switch, in which cells can undergo an epigenetic transition between the white state and the opaque state. The phenotypic switch regulates multiple properties, including biofilm formation, virulence, mating, and fungus-host interactions. Switching between the white and opaque states is associated with many external stimuli, such as oxidative stress, pH, and N-acetylglucosamine, and is directly regulated by the WOR1 transcriptional circuit. The Hog1 stress-activated protein kinase (SAPK) pathway is recognized as the main pathway for adapting to environmental stress in C. albicans. In this work, we first show that loss of the HOG1 gene in a/a and α/α cells, but not α/α cells, results in 100% white-to-opaque switching when cells are grown on synthetic medium, indicating that switching is repressed by the α1/α2 heterodimer that represses WOR1 gene expression. Indeed, switching in the hog1Δ strain was dependent on the presence of WOR1, as a hog1Δ wor1Δ strain did not show switching to the opaque state. Deletion of PBS2 and SSK2 also resulted in C. albicans cells switching from white to opaque with 100% efficiency, indicating that the entire Hog1 SAPK pathway is involved in regulating this unique phenotypic transition. Interestingly, all Hog1 pathway mutants also caused defects in shmoo formation and mating efficiencies. Overall, this work reveals a novel role for the Hog1 SAPK pathway in regulating white-opaque switching and sexual behavior in C. albicans.

Candida albicans is a diploid hemiascomycete yeast that is commonly found in the human gastrointestinal tract (1, 2). However, C. albicans can become a pathogen that attacks multiple organs in the body, including kidney and brain, resulting in life-threatening systemic infections with high mortality rates (3). Phenotypic transitions represent an important mechanism for the striking ability of Candida cells to colonize a wide variety of habitats with diverse properties. For example, under conditions of various environmental signals, the yeast forms of C. albicans can switch to filamentous cells (4–8). The reversible yeast-hypha transition regulated by a core set of transcription factors that mediate its developmental program is complex (7, 9–13). Hyphal formation also plays a major role in biofilm formation, which is closely linked with the propensity to cause infection and is associated with antifungal resistance (14–16).

In addition to the yeast-hypha transition, an important morphological change in C. albicans is the white-opaque switch (17). This epigenetic transition between white and opaque states regulates many aspects of C. albicans biology. In particular, opaque cells are mating competent (18, 19), whereas white cells do not mate but can generate biofilms in response to pheromones (20, 21). The regulation of mating between C. albicans a/a and α/α opaque cells occurs via pheromone signaling between cells of opposite mating types (18, 19, 22, 23). Pheromone responses induce a mitogen-activated protein kinase (MAPK) signal transduction cascade leading to the formation of mating projections (shmoos) and subsequent cell-cell conjugation (21, 24). Furthermore, opaque cells are less susceptible to phagocytosis than white cells due to the lack of secretion of a chemoattractant for leukocytes (25–27), indicating that switching plays a role in host-fungus interactions. Thus, the phenotypic plasticity of C. albicans in response to different host niches is important for its ability to colonize and infect the host.

C. albicans cells typically exist in the default white state, where they are round and form bright, shiny colonies, but can stochastically switch to the opaque state, where cells are more elongated and form darker, flatter colonies (17). The white-opaque switch is regulated by the master regulator Wor1, which acts in concert with several other transcription factors as part of a transcriptional network (28, 29). This network is also controlled by the α1/α2 complex which is produced in α/α heterozygous cells and which represses opaque-cell formation (30), although a recent study showed that some clinical a/α cells have the ability to switch to opaque under certain culture conditions (31). Several environmental factors, including CO2, temperature, oxidative stress, N-acetylglucosamine (NAG), and growth rate, also regulate white-opaque switching (32–34), suggesting that the transition between white and opaque cells is extremely sensitive to external signals and dependent on the expression levels of the master regulator Wor1.

In eukaryotic cells, MAP kinases are responsible for transducing a variety of extracellular signals for growth and differentiation (35, 36). One of them, the high-osmolarity glycerol (Hog1) pathway, is not only responsible for the cellular response to osmotic stress but is also required for responses to UV radiation, temperature, and oxidative stress in the budding yeast (37–39). This pathway is known as the Hog1 stress-activated protein kinase (SAPK) pathway or the Hog1 mitogen-activated protein kinase (MAPK) pathway. Osmosensing triggers Hog1 activation through...
the stimulation of a MAPK kinase kinase (MAPKKK), Ssk2, and a MAPK kinase (MAPKK), Pbs2 (35, 39, 40).

In regard to white-opaque switching, several stresses have been found to promote the switch from white to opaque (32–34). We therefore hypothesized that the Hog1 SAPK pathway of *C. albicans* could be involved in regulating this phenotypic transition. In this study, the results of deletion of *HOG1, PBS2*, and *SSK2* genes in *C. albicans* MTLα/a, MTLα/o, and MTLα/o strains were compared. Mutants in all three strain backgrounds showed high sensitivity to osmotic and oxidative stress, indicating that the roles of the Hog1 SAPK pathway are similar in different cell types. Interestingly, osmotic and oxidative stress, indicating that the roles of the Hog1 pathway mutants exhibit 100% switching from white to opaque but also display shorter shmoos and have reduced mating efficiencies. Overall, our data therefore reveal a novel role for the Hog1 SAPK pathway in white-opaque switching and indicate a further route for the integration of environmental signals into the white-opaque regulatory circuit.

**MATERIALS AND METHODS**

**Media and reagents.** Yeast extract-peptone-dextrose (YPD), Spider, and synthetic complete dextrose (SCD) media used in experiments were prepared as described previously with slight modifications (38, 41).YPD medium, composed of 2% (wt/vol) peptone, 1% (wt/vol) yeast extract, and 2% (wt/vol) glucose, was used for cell growth experiments. Nourseothricin-resistant strains were selected on YPD medium supplemented with 0.2 mg/ml nourseothricin (Werner BioAgents). SCD consisted of 0.7% (wt/vol) yeast nitrogen base without amino acids, 0.17% complete amino acids powder, and 2% (wt/vol) glucose. Synthetic complete (SC) medium was formulated as SCD medium without the addition of glucose. Preparation of Lee’s N-acetylglucosamine (Lee’s NAG), which contained 1.25% N-acetylglucosamine (Alfa Aesar), followed an established protocol (34). SCD, SC, and Lee’s NAG media were used for white-opaque phenotypic transition assays. Spider medium (pH 7.2), containing 1% (wt/vol) mannitol, 1% (wt/vol) nutrient broth, and 0.4% (wt/vol) dipotassium phosphate (Showa Chemical Industry Co.), was used for opaque cell maintenance. Leu*, Arg*, and Arg Leu* media were used to perform a quantitative mating assay. Agar was added to reach a final concentration of 2% (wt/vol) to make solid plates. All chemicals were purchased from Sigma-Aldrich Chemical Co., unless otherwise stated.

**Plasmid and strain constructions.** Strains and primers used in this study are listed in Table 1 and Table 2, respectively.

To generate hog1Δ strains, 5′ and 3′ flanking regions of *HOG1* were amplified using primer pair CH1 and CH2 and primer pair CH3 and CH4. The 5′ and 3′ PCR products were digested with Apal and Xhol and with SacI and SacI and cloned into plasmid pSFSS2A (42) to generate pSFS-HOG1 knockout (KO). This plasmid was linearized with Apal and SacI and then transformed into SC5314 (α/a), RBY717 (α/a), and RBY722 (α/a) to generate strains YL31, YL34, YL8, YL9, YL26, and YL27. To construct the *HOG1* complementary plasmid, primers 10 and 11 were used to amplify the 5′ and 3′ flanking regions of *LEU2* gene, respectively. The PCR products were digested with Apal and Xhol and with SacI and SacI and cloned into plasmid pSFSS2A to construct pSFS-LEU2 KO. The construct was digested with BglII and transformed into RBY717, YL8, and YL9 to create strains YL275, YL276, YL279, and YL280.

To delete the *ARG4* gene in *C. albicans*, the hog1Δ, hog1Δ::HOG1, pbs2Δ, and ssk2Δ mutants, primer pair 114 and 115, and primer pair 116 and 117 were used to amplify the 5′ and 3′ flanking regions of the *ARG4* gene, respectively. The PCR products were digested with Apal and Xhol and with NotI and SacI and cloned into plasmid pSFSS2A to construct pSFSS-ARG4 KO. The construct was digested with Apal and SacI and transformed into RBY717, YL26, YL27, YL35, YL36, YL193, YL194, YL195, and YL196 to generate the YL97, YL98, YL304, YL305, YL328, YL329, YL528, YL529, YL524, and YL525 strains.

To delete the *LEU2* gene, the hog1Δ, hog1Δ::HOG1, pbs2Δ, and ssk2Δ mutants, primer pair 122 and 123, and primer pair 124 and 125 were used to amplify the 5′ and 3′ flanking regions of *LEU2* gene, respectively. The PCR products were digested with Apal and Xhol and with SacI and SacI and cloned into plasmid pSFSS2A to construct pSFS-LEU2 KO. The construct was digested with Apal and SacI and transformed into RBY722, YL8, YL9, YL11, YL12, YL402, YL403, YL400, and YL401 to generate the YL133, YL134, YL191, YL192, YL226, YL227, YL530, YL531, YL526, and YL527 strains.

Opaque cells of each strain were obtained and purified after treatment with Lee’s NAG (Table 1).

**Quantitation of opaque-cell formation.** Overnight cultures of *C. albicans* were plated onto SC, SCD, or Lee’s NAG media supplemented with phloxine B and incubated at 25°C for 4 to 7 days. Opaque colonies were stained red. The white-to-opaque switching efficiency was expressed as follows: (number of opaque colonies)/(total number of colonies) × 100%.

**Sensitivity tests.** Hydrogen peroxide and sodium chloride were purchased from Sigma-Aldrich Co., St. Louis, MO, USA, and MDBio, Inc., Shandong, China, respectively. Determinations of susceptibilities to hydrogen peroxide and sodium chloride were performed by spotting serial 4-fold dilutions of 10°C cells (total volume of 3 μl) from overnight cultures of *C. albicans* onto YPD plates supplemented with 10 mM H2O2 or 1 M NaCl. Plates were incubated at 30°C and examined after 48 h.

**Western blotting.** To examine the level of Hog1 phosphorylation, 200 μl of overnight cultures was diluted into 10 ml of YPD broth and grown for 5 h at 30°C. H2O2 was added to each culture to reach a final concentration of 10 mM, and the reaction mixture was incubated for 15 min after the addition. Samples were collected by centrifugation, suspended in 10 mM Tris-HCl (pH 8.0), and then incubated at 100°C for 10 min to lyse cells. Equal amounts of proteins were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A polyclonal antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and phospho-p38 MAPK (Thr180/Tyr182) antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) were used to detect Hog1 and phos-
### TABLE 1 Strains used in this study

| Strain     | MTL type | Genotype                  | White or opaque | Reference or source |
|------------|----------|---------------------------|-----------------|---------------------|
| SC5314     | a/α      | Wild type                 | White           | 22                  |
| RBY717     | a/a      | ura3::imm434::URA3 iro1::imm434::IRO1 | White           | 22                  |
| RBY722     | α/α      | hog1::HOG1                | White           | 22                  |
| YL8        | a/a      | hog1::HOG1                | White           | This study          |
| YL9        | a/a      | hog1::HOG1                | White           | This study          |
| YL11       | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL12       | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL26       | a/α      | hog1::HOG1                | White           | This study          |
| YL27       | α/α      | hog1::HOG1                | White           | This study          |
| YL31       | a/α      | hog1::HOG1                | White           | This study          |
| YL34       | a/α      | hog1::HOG1                | White           | This study          |
| YL35       | α/α      | hog1::HOG1::HOG1          | White           | This study          |
| YL36       | α/α      | hog1::HOG1::HOG1          | White           | This study          |
| YL38       | a/α      | hog1::HOG1::HOG1          | White           | This study          |
| YL39       | a/α      | hog1::HOG1::HOG1          | White           | This study          |
| YL45       | a/a      | ura3::imm434::URA3 iro1::imm434::IRO1 | Opaque          | This study          |
| YL46       | a/a      | ura3::imm434::URA3 iro1::imm434::IRO1 | Opaque          | This study          |
| YL47       | α/α      | ura3::imm434::URA3 iro1::imm434::IRO1 | Opaque          | This study          |
| YL48       | α/α      | ura3::imm434::URA3 iro1::imm434::IRO1 | Opaque          | This study          |
| YL97       | a/a      | arg4/arg4                 | White           | This study          |
| YL98       | a/a      | arg4/arg4                 | White           | This study          |
| YL133      | α/α      | leu2/leu2                 | White           | This study          |
| YL134      | α/α      | leu2/leu2                 | White           | This study          |
| YL135      | a/a      | wor1/wor1                 | White           | This study          |
| YL136      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL191      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL192      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL193      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL194      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL195      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL196      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL226      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL227      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL234      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL235      | a/a      | hog1::HOG1::HOG1          | White           | This study          |
| YL238      | a/a      | arg4/arg4                 | Opaque          | This study          |
| YL239      | a/a      | arg4/arg4                 | Opaque          | This study          |
| YL259      | a/a      | arg4/arg4                 | Opaque          | This study          |
| YL260      | a/a      | arg4/arg4                 | Opaque          | This study          |
| YL267      | α/α      | hog1::HOG1                | Opaque          | This study          |
| YL268      | α/α      | hog1::HOG1                | Opaque          | This study          |
| YL271      | a/a      | hog1::HOG1::HOG1          | Opaque          | This study          |
| YL272      | a/a      | hog1::HOG1::HOG1          | Opaque          | This study          |
| YL273      | α/α      | leu2/leu2                 | Opaque          | This study          |
| YL274      | α/α      | leu2/leu2                 | Opaque          | This study          |
| YL275      | a/a      | OP4/OP4::OP4p-GFP         | White           | This study          |
| YL276      | a/a      | OP4/OP4::OP4p-GFP         | White           | This study          |
| YL279      | a/a      | hog1::HOG1 OP4/OP4::OP4p-GFP | White           | This study          |
| YL280      | a/a      | hog1::HOG1 OP4/OP4::OP4p-GFP | White           | This study          |
| YL298      | a/a      | hog1::HOG1 OP4/OP4::OP4p-GFP | Opaque          | This study          |
| YL299      | a/a      | hog1::HOG1 OP4/OP4::OP4p-GFP | Opaque          | This study          |
| YL304      | α/α      | hog1::HOG1 arg4/arg4      | White           | This study          |
| YL305      | α/α      | hog1::HOG1 arg4/arg4      | White           | This study          |
| YL328      | α/α      | hog1::HOG1 arg4/arg4      | White           | This study          |
| YL329      | α/α      | hog1::HOG1 arg4/arg4      | White           | This study          |
| YL330      | α/α      | hog1::HOG1 arg4/arg4      | Opaque          | This study          |
| YL331      | α/α      | hog1::HOG1 arg4/arg4      | Opaque          | This study          |
| YL332      | α/α      | hog1::HOG1 arg4/arg4      | Opaque          | This study          |
| YL333      | α/α      | hog1::HOG1 arg4/arg4      | Opaque          | This study          |
| YL400      | α/α      | pbs2/pbs2                 | White           | This study          |
| YL401      | α/α      | pbs2/pbs2                 | White           | This study          |

(Continued on following page)
TABLE 1 (Continued)

| Strain   | MTL type | Genotype | White or opaque | Reference or source |
|----------|----------|----------|-----------------|---------------------|
| YL402    | a/a      | ssk2/ssk2| White           | This study          |
| YL403    | a/a      | ssk2/ssk2| White           | This study          |
| YL404    | a/a      | pbs2/pbs2| White           | This study          |
| YL405    | a/a      | pbs2/pbs2| White           | This study          |
| YL442    | a/a      | ssk2/ssk2| White           | This study          |
| YL443    | a/a      | ssk2/ssk2| White           | This study          |
| YL458    | a/a      | ssk2/ssk2| White           | This study          |
| YL459    | a/a      | ssk2/ssk2| White           | This study          |
| YL469    | a/a      | pbs2/pbs2| White           | This study          |
| YL470    | a/a      | pbs2/pbs2| White           | This study          |
| YL484    | a/a      | pbs2/pbs2| White           | This study          |
| YL485    | a/a      | pbs2/pbs2| White           | This study          |
| YL498    | a/a      | ssk2/ssk2| White           | This study          |
| YL499    | a/a      | ssk2/ssk2| White           | This study          |
| YL524    | a/a      | pbs2/pbs2| White           | This study          |
| YL525    | a/a      | pbs2/pbs2| White           | This study          |
| YL526    | a/a      | pbs2/pbs2| White           | This study          |
| YL527    | a/a      | pbs2/pbs2| White           | This study          |
| YL528    | a/a      | ssk2/ssk2| White           | This study          |
| YL529    | a/a      | ssk2/ssk2| White           | This study          |
| YL530    | a/a      | ssk2/ssk2| White           | This study          |
| YL531    | a/a      | ssk2/ssk2| White           | This study          |
| YL532    | a/a      | ssk2/ssk2| White           | This study          |
| YL533    | a/a      | ssk2/ssk2| White           | This study          |
| YL534    | a/a      | pbs2/pbs2| White           | This study          |
| YL535    | a/a      | pbs2/pbs2| White           | This study          |
| YL536    | a/a      | pbs2/pbs2| White           | This study          |
| YL537    | a/a      | pbs2/pbs2| White           | This study          |
| YL538    | a/a      | pbs2/pbs2| White           | This study          |
| YL539    | a/a      | pbs2/pbs2| White           | This study          |
| YL899    | a/a      | OP4/OP4:OP4p-GFP| White   | This study          |
| YL900    | a/a      | OP4/OP4:OP4p-GFP| White     | This study          |

* All of the strains were SC5314 derived.

Phosphorylated Hog1 proteins, respectively. Blots were developed with Clarity Western ECL substrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Pheromone response assays.** To test formation of mating projections, opaque cells of MTLα/α strains were grown in Spider medium at 25°C overnight. Overnight cultures (500 μl) were diluted into 3 ml of Spider broth, and α-pheromone peptide (GFLRNFGYFEPG; synthesized by GMbioLab Co., Ltd.) was added to reach a final concentration of 10 μg/ml as previously described (43). After 8 h of incubation at 25°C, an Eclipse Ti inverted microscope (Nikon Instruments Inc., Melville, NY, USA) was used to examine the mating projections.

**Quantitative mating assays.** To generate auxotrophic strains, ARG4 and LEU2 genes were deleted in MTLα/a and MTLα/α strains, respectively. Opaque cells were grown in Spider medium at 25°C overnight. Cells (2 × 10^7) of MTLα/a and MTLα/α strains were mixed and placed on Spider plates at 25°C for 48 h. The mixture was collected, diluted, and plated onto selective medium, Arg-, Leu-, and Arg- Leu- plates. Mating efficiencies were calculated by the number of tetraploid colonies on Arg- Leu- plates divided by the total colony number on Arg- or Leu- plates.

**Fluorescence-activated cell sorter (FACS) analysis.** Tetraploid cells from mating progeny were incubated with 95% ethanol for at least 1 h at 4°C and washed with Tris-EDTA (TE) buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8.0). Samples were incubated with RNase A solution (2 mg/ml RNase A, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0) for at least 2 h at 37°C and then with pepsin solution (5 mg/ml pepsin, 55 mM HCl) for 30 min at 37°C and washed with TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5). Samples were incubated with Sytox solution (1 mM Sytox green, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5) for 1 h at 4°C. A BD FACS Canto II system (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) was used to examine the fluorescence of samples.

**Fluorescence microscopy.** White cells and opaque cells of OP4 promoter-driven GFP reporter strains of the wild-type (WT) strain or hog1Δ mutants were mixed, and the expression of fluorescence was examined by the use of an Eclipse Ti inverted microscope (Nikon Instruments Inc., Melville, NY, USA).

**RESULTS**

Deletion of the hog1Δ gene promotes opaque-cell formation. To understand the role of Hog1 in regulation of white-opaque switching, hog1Δ mutants of MTLα/a, MTLα/α, and MTLα/α white cells were plated on synthetic complete (SC), synthetic complete dextrose (SCD), and N-acetylglucosamine (NAG) media supplemented with phloxine B to test their switching frequencies. Figure 1A shows that both a/a and α/α colonies of hog1Δ mutants switched to the opaque state (100% switching) when grown on SC medium, whereas α/α cells remained in the white state. Reintegration of the HOGL1 gene in each mutant restored the WT phenotype on SC medium, with colonies remaining in the white state.

Interestingly, the high rates of switching observed with hog1Δ mutants were not observed on medium supplemented with glucose (SCD) (Fig. 1B). The high rates of switching were observed with hog1Δ mutants from both homozygous strain backgrounds also showed 100%
opaque switching rates, whereas the WT and complemented mutant strains showed intermediate (63% to 85%) levels of opaque-cell formation (Fig. 1C). To test whether opaque-looking cells were expressing opaque state-specific genes, we expressed the promoter fused to GFP in both wild-type and each SAPK component exhibited marked reductions in the length of the WT homozygous strains and not in the WT heterozygous strain.

**Functions of the SAPK components are well conserved in both homozygous and heterozygous strains.** The Hog1 SAPK pathway is necessary for resistance to osmotic and oxidative stresses. However, previous studies were performed in *C. albicans* a/α heterozygous cells (45, 46). We therefore compared and tested the roles of SAPK pathway components in both the heterozygous and homozygous strain backgrounds. As shown in Fig. 4A, mutants of each SAPK component were highly sensitive to both osmotic and oxidative stress in a/α, a/a, and a/α cells. Western blotting also revealed that the function of SAPK in *C. albicans* is conserved and that Hog1 activation is regulated by Ssk2 and Pbs2 in *MTL* homozygous strains, as it is in heterozygous strains (Fig. 4B).

**Deletion of HOGL, PBS2, and SSK2 genes results in shorter mating projections.** *C. albicans* strains must undergo several developmental steps before having sex (47). Most isolates are diploid a/a strains and have to undergo homoygosis to generate a/α cells. These homozygous cells must then switch from white to opaque to become mating competent (18, 19). Phenomone treatment of *C. albicans* opaque cells activates mating responses and leads to the formation of mating projections (21–23, 43). We further examined the mating characteristics of opaque cells obtained from holog1, pbs2Δ, and ssk2Δ mutants. Surprisingly, mutants of each SAPK component exhibited marked reductions in the length of their mating projections (10 to 12 μm) relative to the length

---

**TABLE 2 Oligonucleotides used in this study**

| Oligonucleotide | Sequence (5′–3′) |
|-----------------|-----------------|
| CH1             | GGAGCGGGGCAGCCAGGAACAGACTTTGAAAC |
| CH2             | GGAGCGGCTGAGTTGGTATGTTATATTTCAGCCGAG |
| CH3             | GGAGGCCGCGCTCTCAAAAATACAGCTAGCAATTATAG |
| CH4             | GGAGGGGAAGCTCTGCTCGCTTCAATTTGAAAAAG |
| 1               | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 2               | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 3               | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 4               | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 5               | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 6               | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 7               | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 8               | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 9               | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 10              | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 11              | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 12              | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 13              | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 14              | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 15              | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 16              | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 17              | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 18              | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |
| 19              | GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG |

---

**Oligonucleotides used in this study**

- CH1: GGAGCGGGGCAGCCAGGAACAGACTTTGAAAC
- CH2: GGAGCGGCTGAGTTGGTATGTTATATTTCAGCCGAG
- CH3: GGAGGCCGCGCTCTCAAAAATACAGCTAGCAATTATAG
- CH4: GGAGGGGAAGCTCTGCTCGCTTCAATTTGAAAAAG
- 1: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 2: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 3: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 4: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 5: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 6: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 7: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 8: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 9: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 10: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 11: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 12: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 13: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 14: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 15: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 16: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 17: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 18: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG
- 19: GCCGAGCTACGAGCTAGGTTGGTATGTTATATTTCAGCCGAG

---

**Hog1 SAPK in Controlling White-Opaque Switching**

- Wor1. We observed that holog1 a/α cells remain locked in the white phenotype (Fig. 1). This is presumably due to the role of the a1/a2 heterodimer in inhibiting WOR1 expression (30), thereby impeding opaque-cell formation. To determine whether WOR1 is necessary for opaque-cell formation in holog1 mutants, holog1 wor1Δ double-knockout strains were created. In contrast to the holog1 mutant, the holog1 wor1Δ mutant lacked the ability to switch to opaque when plated on SC medium (Fig. 2).

- Both Pbs2 and Ssk2 are involved in white-opaque switching. The SAPK signaling cascade consists of three serine/threonine protein kinases: MAPKKK (Ssk2), MAPKK (Pbs2), and MAPK (Hog1) (39, 40, 44). Ssk2 phosphorylates Pbs2, which in turn phosphorylates and activates Hog1. To determine whether Pbs2 and Ssk2 also regulate the white-opaque transition, mutants were constructed and plated on SC medium. Similarly to the holog1 mutant (Fig. 1), the pbs2Δ and ssk2Δ mutants exhibited 100% opaque-cell formation on SC medium supplemented with phloxine B (Fig. 3). Once again, switching occurred only in MTL homozygous strains and not in the MTL heterozygous strain.
seen with the wild-type strain (24 μm) (Fig. 5). These results indicate that although deletion of HOG1, PBS2, and SSK2 promotes opaque-cell formation, these mutants are less capable of forming mating projections of normal lengths.

FIG 2 The lack of WOR1 gene strongly inhibits Hog1-mediated white-to-opaque switching. White cells of the MTLα/a WT strain, hog1Δ mutants, and hog1Δ wor1Δ double mutants were plated onto SC plates supplemented with phloxine B. Images were taken after a 7-day incubation at 25°C. Opaque cells were stained red. The total numbers of cells examined for each strain were 1,000 to 1,200. Switching frequency was expressed as a percentage of the mean ± SD and is shown below each image. "<" indicated that no opaque or opaque-sectored colony was observed.

FIG 3 MAPKKK Ssk2 and MAPKK Pbs2 are involved in the regulation of white-opaque switching. White cells of each mutant were plated onto the SC medium supplemented with phloxine B and incubated at 25°C for 7 days. White-to-opaque switching efficiencies were expressed as percentages of averages ± standard deviations (SD) and are shown below each image. At least 1,000 colonies were examined. "<" indicates that no opaque-cell formation was observed.
Hog1 SAPK in Controlling White-Opaque Switching

**DISCUSSION**

*C. albicans* is the most important fungal pathogen in humans, where many infections arise and the difficulties for treatment are due to its inherent flexible lifestyle and ability to avoid the immune system. In addition to the yeast-hypha transition, epigenetic variation via white-opaque switching is perhaps the best example of morphological plasticity. In this work, we first demonstrated that the Hog1 SAPK signaling cascade is involved in regulating the white-opaque epigenetic transition. Inactivation of SSK2, PBS2, and HOG1 genes in MTLα/a and MTLα/α cells led to high sensitivity to osmotic and oxidative stresses and also promoted switching *en masse* to the opaque cell state under certain culture conditions. These results indicate that although the SAPK in *C. albicans* is evolutionarily well conserved among eukaryotes, the biological functions of each component kinase are highly dependent on the lifestyles of the species and their environment.

Given that Hog1 can impact white/opaque phenotypic states, we were curious whether the postmodification states of Hog1 differ between white and opaque cells. However, cells of the two types showed similar levels of phosphorylation of the Hog1 protein (data not shown). Consistent with previous results, expression levels of Hog1 were also comparable between white and opaque cells (48). Nevertheless, we cannot rule out the possibility that changes in gene expression levels or postmodification states of Hog1 occur during the white-to-opaque switching process.

*C. albicans* Hog1 SAPK is activated by many environmental cues, including osmotic and oxidative stimuli, heavy metal, glucose, and cationic peptides (45, 49–51). Two of them, oxidative-stress levels and glucose concentrations, have been reported to be involved in opaque-cell formation and its stability (32, 34, 52). Our studies highlighted that deletion of *HOG1* led to switching of white cells to the opaque state on SC and NAG media. These observations could be because removal of *HOG1* causes cells to be more sensitive to internal and external oxidative microenvirom-

---

**FIG 4** The roles of SSK2, PBS2, and HOG1 in regard to osmotic and oxidative stresses are indistinguishable between homozygous and heterozygous strains of *C. albicans*. (A) Serially diluted cells of MTLα/a, MTLα/α, and MTLα/α strains of the wild-type, ssk2Δ, pbs2Δ, and hog1Δ mutant strains were spotted onto Spider medium supplemented with 1 M NaCl or 10 mM hydrogen peroxide. (B) Phosphorylation levels of Hog1p were analyzed in MTLα/a, MTLα/α, and MTLα/α strains of ssk2Δ and pbs2Δ mutants. H2O2 (10 mM) was added to exponentially growing cultures, and samples were collected after a 15-min treatment. Anti-Hog1 antibody and phospho-p38 (Thr180/Tyr182) antibody were used to detect Hog1 and phospho-Hog1 (Hog1p), respectively.

**FIG 5** ssk2Δ, pbs2Δ, and hog1Δ mutants exhibited shorter mating projections. (A) White cells and opaque cells of the hog1Δ, ssk2Δ, pbs2Δ, and WT strains were challenged with or without α-factor for 8 h at 25°C. The cells were then imaged with a Nikon Eclipse Ti microscope. (B) At least 50 cells were analyzed to measure the projection length after an 8-h pheromone treatment. The mating projection length of each strain is expressed as the mean ± SD. MF-α, *C. albicans* pheromone peptide. *, P < 0.001.

ssk2Δ, pbs2Δ, and hog1Δ mutants exhibit reduced mating efficiencies. Given that ssk2Δ, pbs2Δ, and hog1Δ strains exhibited shorter mating projections (Fig. 5), it is possible that these mutants would also have defects in mating. Opaque MTLα/a and MTLα/α cells containing different selection markers were constructed as described in Materials and Methods. Quantitative mating assays were performed, and the results are shown in Fig. 6A. As expected on the basis of the defective mating responses, the mating efficiencies of ssk2Δ, pbs2Δ, and hog1Δ mutants were 0.6%, 1.6%, and 2.7%, respectively (Fig. 6A). In contrast, the wild-type strain showed a mating efficiency of 16.7% (Fig. 6A). All the mating progeny were confirmed by PCR and FACS analysis to be tetraploid MTLα/α cells (Fig. 6B and C).
ments that drive cells to the opaque state. Furthermore, it is possible that a transcription factor regulated by Hog1 impacts Wor1 expression and switching frequencies (Fig. 7). Indeed, our studies demonstrated that Wor1 plays a crucial role in the morphological change in hog1Δ mutants, as no white-to-opaque switching was observed in MTLa/a cells or in hog1Δ wor1Δ mutants. Whether genetic control (induction of Wor1 expression) or environmental control (increased sensitivity to stress conditions) or a combination of these factors induces switching remains an open question. Despite high switching rates of hog1Δ mutants on SC and NAG media, we have not observed opaque-colony formation on SCD plates. Similar observations have been reported to show that the presence of glucose (dextrose) inhibited opaque-cell formation and its stability (34, 52). However, the exact mechanism by which glucose or its metabolism influences phenotypic switching or the stability of opaque cells remains obscure (Fig. 7).

The pheromone response for C. albicans is mediated by Cph1, a downstream target of Cek1/Cek2 MAP kinases (21). Deletion of the CPH1 gene resulted in loss of pheromone-stimulated cell adhesion in white cells and mating-projection formation in opaque cells (21). Despite loss of Hog1 SAPK components promoting opaque-cell formation, upon pheromone challenge, hog1Δ, pbs2Δ, and ssk2Δ mutants exhibited projections that were shorter than those of the wild-type strain. Apparently, the pheromone response in these mutant cells is decreased relative to the level in the wild type. These mutants also exhibited a significant decrease in the level of sexual mating. It is clear that C. albicans cells have to switch to the opaque state before having sex and that the mechanisms of the switch and mating response are disparate (22, 53).

For example, when the pheromone receptor Ste2 or Cph1 is removed, cells are still able to switch to the opaque state but cannot respond to pheromones and cannot undergo α/α mating (21).

Regulatory interactions between the Hog1 and mating pathways in Saccharomyces cerevisiae have been reported (54–60). Analyses of the antagonistic manners of the potential interactions of two MAPKs were conducted via examination of the insulation mechanism, a physical separation of the shared components of two cascades (54, 59, 61). Since the Hog1 and mating pathways share several components, including Cdc2, Ste20, Ste50, and Ste11, it is therefore possible that Hog1-mediated phosphorylation might cause the conformational changes of shared components to control cell fate (59, 61). The Ste5 and Pbs2 scaffold proteins exclusively required for the mating and hyperosmotic responses, respectively, also appear to regulate and maintain the response specificity (61). Additionally, pathway cross-inhibition has been previously addressed (61). When Hog1 is deleted or inhibited, mating is promoted under conditions of hyperosmotic stress (54, 57). A recent study further indicated that activation of Hog1 can (i) phosphorylate Ste50, leading to the downregulation...
of mating signal, and (ii) repress the Fus3 response shown by Rck2, a protein kinase of the cell cycle checkpoint (60). It was previously noted that S. cerevisiae contains two osmosensors (Sho1 and Sln1) for Hog1 activation (39). The shared components of Cdc2, Ste20, Ste50, and Ste11 in S. cerevisiae cooperate only with the Sho1-mediated branch (39, 60). Interestingly, although a conserved mechanism by which external signals are sensed and relayed to the SAPK pathway has been observed in C. albicans, mechanisms for activation of the SAPK pathways in the budding yeast and C. albicans have diverged. Indeed, Hog1 SAPK signaling in C. albicans is entirely dependent on a sole osmosensor, Sln1, whereas the Sho1 branch in C. albicans plays no role in Hog1 activation (39, 46). These results therefore suggest a more interesting issue: the potential mechanisms by which the Hog1 SAPK pathway impacts mating in C. albicans.

Taking the data together, white and opaque cells differ in many aspects, as they exhibit different colony morphologies and different mating efficiencies, colonize different niches in the host, and have different interactions with host immune cells. Our work has identified a novel role for the Hog1 SAPK pathway in mediating white-opaque switching. Whether there is a niche in the human body that inhibits Hog1 signaling, leading to increased opaque-cell formation and potential escape from the immune system, is therefore an interesting issue. Future studies will investigate the roles of several key upstream regulatory factors and downstream targets of the Hog1 SAPK pathway to determine if these factors function in regulating this unique phenotypic transition in the human fungal pathogen C. albicans.

ACKNOWLEDGMENTS

We thank Richard Bennett for stains and plasmids. We also thank Richard Bennett and members of his laboratory for critical reading of and comments on the manuscript. We are grateful to the staff of Technology Commons, College of Life Science, National Taiwan University, for the help with the BD FACSanto II system. This research was supported by NSC102-2320-B-002-027-MY3, MOST 103-2628-B-002-003-MY3, and NTU 103R7787 from the National Science Council, the Ministry of Science and Technology, and National Taiwan University, respectively.

REFERENCES

1. Gargas A, Taylor JW. 1995. Phylogeny of discomycetes and early radiations of the apostle Ascomycotina inferred from SSU rDNA sequence data. Exp. Mycol. 19:7–15. http://dx.doi.org/10.1006/emyc.1995.1002.
2. Hedges SB. 2002. The origin and evolution of model organisms. Nat. Rev. Genet. 3:838–849. http://dx.doi.org/10.1038/nrg929.
3. Ruhneke M, Maschmeyer G. 2002. Management of mycoses in patients with hematologic disease and cancer – review of the literature. Eur. J. Med. Res. 7:227–235.
4. Buffo J, Herman MA, Soll DR. 1984. A characterization of pH-regulated dimorphism in Candida albicans. Mycopathologia 89:21–30. http://dx.doi.org/10.1007/BF00436698.
5. Mardon D, Balish E, Phillips AW. 1969. Control of dimorphism in a biochemical variant of Candida albicans. J. Bacteriol. 100:701–707.
6. Simonetti N, Strippoli V, Cassone A. 1974. Yeast mycelial conversion induced by N-acetyl-D-glucosamine in Candida albicans. Nature 250:344–346. http://dx.doi.org/10.1038/250344a0.
7. Sudbery PE. 2011. Growth of Candida albicans hyphae. Nat. Rev. Microbiol. 9:737–748. http://dx.doi.org/10.1038/nrmicro2636.
8. Taschdjian CL, Burchall JJ, Kozinn PJ. 1960. Rapid identification of Candida albicans by filamentation on serum and serum substrates. AMA J. Dis. Child. 99:212–215. http://dx.doi.org/10.1001/archpedi.1960.02070030214011.
9. Sudbery P, Gow N, Berman J. 2004. The distinct morphogenetic states of Candida albicans. Trends Microbiol. 12:317–324. http://dx.doi.org/10.1016/j.tim.2004.05.008.
10. Stoldt VR, Sonneborn A, Leuker CE, Ernst JF. 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen Candida albicans, is a member of a conserved class of bZIP proteins regulating morphogenetic programs in fungi. EMBO J. 16:1982–1991. http://dx.doi.org/10.1093/emboj/16.18.1982.
11. Si H, Hernday AD, Hirakawa MP, Johnson AD, Bennett RJ. 2013. Candida albicans white and opaque cells undergo distinct programs of filamentous growth. PLoS Pathog. 9:e1003210. http://dx.doi.org/10.1371/journal.ppat.1003210.
12. Shapiro RS, Vuppuluri P, Zaas AK, Collins C, Senn H, Perfect JR, Heitman J, Coven LE. 2009. Hog9 orchestrates temperature-dependent Candida albicans morphogenesis via Ras1-PKA signaling. Curr. Biol. 19:621–629. http://dx.doi.org/10.1016/j.cub.2009.03.017.
13. Shapiro RS, Robbins N, Coven LE. 2011. Regulatory circuitry governing fungal development, drug resistance, and disease. Mol. Biol. Rev. 75:213–267. http://dx.doi.org/10.1128/MMBR.00045-10.
14. Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, Hernday AD, Tuch BB, Andes DR, Johnson AD. 2012. A recently evolved transcriptional network controls biofilm development in Candida albicans. Cell 148:126–138. http://dx.doi.org/10.1016/j.cell.2011.10.048.
15. Soll DR. 2002. Management of mycoses in patients infected with Candida albicans. Mycol. Pract. 2002. The origin and evolution of model organisms. Nat. Rev. Genet. 3:838-849. http://dx.doi.org/10.1038/nrg929.
16. Ruhneke M, Maschmeyer G. 2002. Management of mycoses in patients with hematologic disease and cancer – review of the literature. Eur. J. Med. Res. 7:227-235.
expression of WOR1, a master regulator of white-opaque switching in *Candida albicans*. Proc. Natl. Acad. Sci. U. S. A. 103:12813–12818. http://dx.doi.org/10.1073/pnas.0605270103.

31. Xie J, Tao L, Nobile CJ, Tong Y, Guan G, Sun Y, Cao C, Hernandy AD, Johnson AD, Zhang L, Bai FY, Huang G. 2013. White-opaque switching in natural *MTLA* isolates of *Candida albicans*: evolutionary implications for roles in host adaptation, pathogenesis, and sex. PLoS Biol. 11: e1001525. http://dx.doi.org/10.1371/journal.pbio.1001525.

32. Alby K, Bennett RJ. 2009. Stress-induced phenotypic switching in *Candida albicans*. Mol. Biol. Cell 20:3178–3191. http://dx.doi.org/10.1016/j.micinf.2009-01-0040.

33. Huang G, Srikanth T, Sahni N, Yi S, Soll DR. 2009. MAPK cell-cycle regulation in *Saccharomyces cerevisiae* and *Candida albicans*. Future Microbiol. 5:1125–1141. http://dx.doi.org/10.2217/fmb.09.27.

34. Xu JR. 2000. MAP kinases in fungal pathogens. Fungal Genet. Biol. 31:137–152. http://dx.doi.org/10.1006/lgdb.2000.1237.

35. Alby K, Bennett RJ. 2009. Stress-induced phenotypic switching in *Candida albicans* by mutation of a STE11 homolog. Science 266:1723–1726. http://dx.doi.org/10.1126/science.7992058.

36. Smith DA, Morgan BA, Quinn J. 2010. Stress signalling to fungal stress-activated protein kinase pathways. FEMS Microbiol. Lett. 306:1–8. http://dx.doi.org/10.1111/j.1574-6968.2010.01937.x.

37. Liu H, Kohler J, Fink GR. 1994. Suppression of hyphal formation in *Candida albicans* by mutation of a STE12 homolog. Science 266:1723–1726. http://dx.doi.org/10.1126/science.7992058.

38. Smith MA, Madhani HD. 2004. Principles of MAP kinase signaling pathways with common components. Sci. Signal. 283:ra184–ra186. http://dx.doi.org/10.1126/sci.signal.2003269.

39. Bruce CR, Quinn J. 2006. Analysis of mitogen-activated protein kinase pathways in *Saccharomyces cerevisiae*. Methods in enzymology, vol 194. 127–149. http://dx.doi.org/10.1016/0076-6879(86)07738-4.

40. Reuss O, Vik A, Kolter R, Morschhauser J. 2004. The SAT1 flipper, an optimized tool for gene disruption in *Candida albicans*. Genes & Development 18:119–127. http://dx.doi.org/10.1101/gad.2004.06.021.

41. Lin CH, Choi A, Bennett RJ. 2011. Defining pheromone-receptor signaling in *Candida albicans* and related asexual *Candida* species. Mol. Biol. Cell 22:4918–4930. http://dx.doi.org/10.1091/mbc.E11-09-0749.

42. de Dios CH, Roman E, Monge RA, Pla J. 2010. The role of MAPK signal transduction pathways in the response to oxidative stress in the fungal pathogen *Candida albicans*: implications in virulence. Curr. Protein Pept. Sci. 12:69–76. http://dx.doi.org/10.2174/138920310794557655.

43. The Hog1 mitogen-activated protein kinase pathway. Eukaryot. Cell 12:2874–2886. http://dx.doi.org/10.1011/gad.12.18.2874.

44. Harris K, Lamson RE, Nelson B, Hughes TR, Marton MJ, Roberts CJ, Boone C, Pryciak PM. 2001. Role of scaffolds in MAP kinase pathway specificity revealed by custom design of pathway-dedicated signaling proteins. Curr. Biol. 11:1815–1824. http://dx.doi.org/10.1016/S0960-9822(01).00567-X.

45. O'Rourke SM, Herskowitz I. 1998. The Hog1 MAPK prevents cross talk between the HOG and pheromone response MAPK pathways in Saccharomyces cerevisiae. Genes Dev. 12:2874–2886. http://dx.doi.org/10.1101/gad.12.18.2874.

46. Westfall PJ, Thorner J. 2006. Analysis of mitogen-activated protein kinase signaling specificity in response to hyperosmotic stress: use of an analog-sensitive HOG1 allele. Eukaryot. Cell 5:1215–1228. http://dx.doi.org/10.1128/EC.00037-06.

47. Brown AJ. 2009. Glucose promotes stress resistance in the fungal pathogen *Candida albicans*. Mol. Biol. Cell 20:4845–4855. http://dx.doi.org/10.1038/mbp.2009.10057655.

48. Smith DA, Nicholls S, Morgan BA, Brown AJ, Quinn J. 2004. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. Mol. Biol. Cell 15:4179–4190. http://dx.doi.org/10.1091/mbc.E04-01-0181.

49. Vylkova S, Jang WS, Li W, Nayyar N, Edgerton M. 2007. Histatin 5 initiates osmotic stress response in *Candida albicans* via activation of the Hog1 mitogen-activated protein kinase pathway. Eukaryot. Cell 6:1876–1888. http://dx.doi.org/10.1011/ECC.00039-07.

50. Liang et al. 2005. Histatin 5 elicits caspase-dependent apoptosis in RAW264.7 cells. J. Immunol. 174:5943–5950. http://dx.doi.org/10.1111/j.1600-065X.2005.04099.x.

51. Schwartz MA, Madhani HD. 2004. Principles of MAP kinase signaling specificity in *Saccharomyces cerevisiae*. Annu. Rev. Genet. 38:725–748. http://dx.doi.org/10.1146/annurev.genet.38.070303.112634.