Sac7 and Rho1 regulate the white-to-opaque switching in *Candida albicans*

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*Candida albicans* cells homozygous at the mating-type locus stochastically undergo the white-to-opaque switching to become mating-competent. This switching is regulated by a core circuit of transcription factors organized through interlocking feedback loops around the master regulator Wor1. Although a range of distinct environmental cues is known to induce the switching, the pathways linking the external stimuli to the central control mechanism remains largely unknown. By screening a *C. albicans* haploid gene-deletion library, we found that *SAC7* encoding a GTPase-activating protein of Rho1 is required for the white-to-opaque switching. We demonstrate that Sac7 physically associates with Rho1-GTP and the constitutively active Rho1<sup>G18V</sup> mutant impairs the white-to-opaque switching while the inactive Rho1<sup>D124A</sup> mutant promotes it. Overexpressing *WOR1* in both *sac7*Δ/Δ and *rho1G18V* cells suppresses the switching defect, indicating that the Sac7/Rho1 module acts upstream of Wor1. Furthermore, we provide evidence that Sac7/Rho1 functions in a pathway independent of the Ras/cAMP pathway which has previously been positioned upstream of Wor1. Taken together, we have discovered new regulators and a signaling pathway that regulate the white-to-opaque switching in the most prevalent human fungal pathogen *C. albicans*.

The fungus *Candida albicans* (*Ca*) is usually a harmless member of the human microbiota colonizing diverse host niches such as the oral cavity and gastrointestinal and urogenital tracts. *C. albicans* is also an opportunistic pathogen responsible for millions of mucosal infections every year in otherwise healthy individuals and can cause life-threatening systemic infections in immunocompromised patients with high mortality rates. The ability to switch between different morphological forms, such as yeast and hyphae, is thought to be a critical determinant of *C. albicans* virulence.

Another well-studied morphological change seen in *C. albicans* is the white-to-opaque phenotypic transition. White cells are oval-shaped and form white, hemispherical colonies with a smooth surface typical of the ones formed by standard *C. albicans* strains, while opaque cells are elongated and generate large, flat gray colonies. Opaque cells often have a giant vacuole in the cytoplasm and possess pimple-like structures on the cell wall, and opaque colonies become pink/red when grown in the presence of Phloxine B (PB), a dye routinely used to differentiate opaque from white colonies.

The biological role of the white-to-opaque transition is intimately related to mating, a rare event in the life cycle of *C. albicans* but important for generating genetic diversity for adaption to changing environments. Only opaque cells are capable of mating. The vast majority of natural *C. albicans* isolates are diploids and heterozygous at the mating-type locus (*MTLa*/*α*) which encodes an a1-α2 transcriptional co-repressor that keeps *C. albicans* cells in the white state. To mate, the a/α white cells must first undergo homozygosis at the *MTL* to produce a/a or α/α cells, enabling the switch to the opaque state. A low level of spontaneous white-to-opaque switching occurs and has been shown to be required for the formation of *MTL*-homozygous white cell “sexual” biofilms which facilitate the mating of minority opaque cells. Moreover, this phenotypic transition may also assist host commensalism and pathogenesis. For example, opaque cells have lost the capacity to release a potent

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C. albicans the switching defect of the RAS1 defects in exacerbated deletion mutants and the strain expressing the active Rho1, while deletion of SAC7 of biofilm formation and polarized growth32,33. As mating normally occurs between haploid cells in most eukary-

levels of CO2 induce the white-to-opaque switching and stabilize the opaque phenotype24. Acidic pH increases the and transduce external signals to the central transcriptional control of the white-to-opaque switching is likely to

stimulated switching in both white-to-opaque and opaque-to-white directions 27. Genotoxic stress generated by chemical treatment (MMS and HU) or gene deletion (RAD51 and RAD52) and oxidative stress produced by hydrogen peroxide all induce efficient white-to-opaque switching26. A largely unanswered question is how these diverse environmental signals cause corresponding changes in the activity of the central transcription network to effect the white-to-opaque-to-hypha transition. The Ras/cAMP signaling cascade is a primary signal transduction pathway that mediates a range of cellular responses, including the white-to-opaque and yeast-hypha transition, to various environmental signals26. The adenylyl cyclase Cyr1, a large protein and an essential component of the Ras1/cAMP pathway, carried multiple signal sensors along its length39. However, the mechanisms that sense and transduce external signals to the central transcriptional control of the white-to-opaque switching is likely to be more much more complex than what is currently known1.

Molecular genetics is a powerful tool for the discovery of mechanisms that control a biological process. However, standard genetic screens had largely been inapplicable in C. albicans because of its diploid genome. This situation has begun to improve with the recent discovery of haploid C. albicans and the construction of tool strains30,31. Although the haploids were generated from heterozygous diploids through concerted chromosome loss and hence have a different genetic background from their parents, they inherited the defining characteristics of standard diploid C. albicans including the yeast-hypha transition, which we were particularly suitable for uncovering new mechanisms that control this biological event.

Previously, we constructed a haploid C. albicans gene deletion library covering most uncharacterized GTPases and their regulators listed in Candida Genome Database32. A significant number of the genes are related with Rho GTases which are members of the Ras superfamily of small GTP-binding proteins34. Rho GTases are molecular switches, cycling between an active GTP-bound form and an inactive GDP-bound form. GDP/GTP exchange factors (GEFs) activate Rho GTases by promoting the formation of Rho1-GTP34,35, while GTase-activating proteins (GAPs) inactivate Rho GTases by enhancing GTP hydrolysis34,36. Rho GTases are often positioned at the top of signal transduction pathways and interact with multiple downstream effectors to orchestrate various cellular processes important for cellular morphogenesis such as cytokinetics, gene transcription, cell division, polarity establishment and maintenance, and membrane trafficking. Several GTases and their regulators have been reported to control the yeast-to-hypha transition, a trait critical for the virulence of C. albicans37,38. In this study, we screened the same haploid mutant library to look for new regulators of the white-to-opaque transition. We found that deletion of SAC7, a Rho1 GAP, significantly blocked the white-to-opaque switching in both haploid and MTL-homozygous diploid cells. Consistent with its role as a Rho1 GAP, we detected physical interaction of Sac7 with GTP-Rho1 but not GDP-Rho1. Expression of a constitutively active Rho1 caused a switching defect similar to that caused by the SAC7 deletion. Overexpression of WOR1 rescued the switching defects in SAC7 deletion mutants and the strain expressing the active Rho1, while deletion of RAS1 exacerbated the switching defect of the sac7Δ/Δ mutant. Our results indicate that Sac7/Rho1 acts upstream of Wor1 and independent of the Ras1/cAMP pathway. Thus, our findings have identified Sac7 and Rho1 as key elements of a novel signaling pathway that regulates the white-to-opaque transition in C. albicans.

Results
Haploid sac7Δ mutant is defective in the white-to-opaque switching. Using the stable haploid strain GZY903, we constructed a gene deletion library of GTases and their regulators37, most of which had not been characterized. To identify new regulators of the white-to-opaque switching, we screened the library for mutants unable to do so under an inducing condition (YPD, pH 6.0). Following a published protocol35, we spread haploid white cells on pH 6.0 YPD plates supplemented with PB and incubated the plates in the dark at 25°C for 6–7 days. While nearly all wild-type (WT) colonies became pink, the majority of the colonies from 5 mutants ( sac7Δ, bem2Δ, trs120Δ, yip4Δ, and nag1Δ) remained white, indicating defects in the white-to-opaque switching (Fig S1). We chose sac7Δ for further analyses, because SAC7 encodes a putative GTase-activating protein for the important signaling molecule Rho1.

Under the inducing condition, 88.0% (n = 150) of the colonies of the WT haploid GZY903 (GZY903 + URA3) cells were stained pink, some with red dots or sectors, while the rest were white (Fig. 1a). Microscopic examination...
revealed that the white colonies contained only white cells (wh) which were round or ellipsoidal (Fig. 1a, W1). In contrast, the majority of cells from the pink/red colonies exhibited characteristics of typical opaque cells (op) which were large and elongated with a huge cytoplasmic vacuole (Fig. 1a, R1). Under the same inducing condition, only 37.3% (n = 220) of the sac7Δ colonies were pink/red (Fig. 1a). Cells from white sac7Δ colonies were all white cells (Fig. 1a, W2) while those in the pink/red colonies were a mixture of white and opaque cells (Fig. 1a, R2). The reduced number of PB-stained colonies of the sac7Δ mutant suggests that Sac7 may play a role in the white-to-opaque switching.

As we had noticed that cells of some C. albicans strains were stained pink by PB even under non-inducing condition (YPD, pH 7.0) and the cells had a typical yeast morphology, we sought to find a more reliable way to distinguish opaque and white cells. We used the promoter of the opaque-specific gene OP4 to control the expression of C. albicans codon-optimized dTomato (dTMT)39, an exceptionally bright red fluorescent protein ideal for live cell imaging40. We introduced this construct into both GZY903 (WT) and the sac7Δ mutant to generate
GZY803 + URA3 + dTMT and sac7Δ + URA3 + dTMT strains, respectively, and grew them under the inducing condition. When examined under a fluorescence microscope, the majority of the GZY803 + URA3 + dTMT colonies strongly expressed dTomato as bright sectors or punctate dots, and only a few colonies/sectors lacked detectable signal and remained evenly dim (Fig. 1b). Further examination of GZY803 + URA3 + dTMT colonies revealed that cells from the dim colonies were typical white cells without detectable dTomato signals (Fig. 1b, W1). In contrast, the majority of cells from the bright sectors were characteristic of opaque cells emitting strong red fluorescence (Fig. 1b, R1); and cells from the colonies with punctate dots were a mixture of white and opaque cells (Fig. 1b, R2). Therefore, expression of dTomato from the OP4 promoter allows us to distinguish opaque from white cells not only on the colony level but also the cellular level. Under the same inducing condition, fewer colonies of sac7Δ + URA3 + dTMT expressed dTomato and the signals were much weaker overall (Fig. 1b, W2 and R3), confirming that the sac7Δ mutant is defective in the white-to-opaque switching.

The white-to-opaque switching defect of sac7Δ was rescued by SAC7 and suppressed by WOR1 overexpression. To confirm that the loss of SAC7 was indeed responsible for the white-to-opaque switching defect in the haploid sac7Δ mutant, we re-introduced a WT SAC7 gene to generate a rescued strain sac7Δ + SAC7 + dTMT. After the induction, 86.6% (n = 209) of WT haploid (GZY803 + URA3 + dTMT), GZY1153 (sac7Δ + SAC7 + dTMT), and GZY1130 (sac7Δ + WOR1OE + dTMT) were spread onto YPD plates (pH 6.0, +PB) and incubated at 25 °C in the dark for 7 days. The plates were examined under a fluorescent microscope to capture colony images through the TBF channel and detect dTomato signals through the RFP channel with the same exposure time. The percentage of dTomato-expressing colonies of each strain was calculated.
Wor1 is the master regulator of the white-to-opaque switching. While deletion of Wor1 blocks the formation of opaque cells, overexpression of Wor1 converts all cells to opaque cells even in MTL-heterozygous cells. To investigate the epistatic relationship between Sac7 and Wor1, we generated a strain that overexpressed Wor1 (WOR1OE) from the tetracycline-suppressible (Tet-off) promoter in the sac7Δ background. Under the inducing condition, all colonies (n = 133) of the sac7Δ + WOR1OE strain produced strong dTomato signals (Fig. 2), indicating the suppression of the switching defect of the sac7Δ mutant by Wor1 overexpression.

The data, therefore, indicate that Sac7 functions upstream of Wor1 in the regulation of the white-to-opaque transition.

Diploid sac7Δ/Δ mutant was also defective in the white-to-opaque switching. To examine whether Sac7 is also involved in the white-to-opaque switching in diploid cells, we generated a sac7Δ/Δ mutant in WUM5A, an MTL-homozygous strain derived from WO-1 and routinely used in the studies of the white-to-opaque transition. To induce the switching, the diploid cells were first grown on GMM plates at 25 °C for 3 days and then collected in water before plating onto Lee's GlcNAc plates (pH 6.0, +PB) for incubation in the dark at 25 °C for 6 days. Under this inducing condition, the majority of the WUM5A colonies were entirely or partly stained red by PB with only a few white colonies (Fig. 3a). Morphological examination confirmed that cells from the red colonies were typical opaque cells with pimples on cell surface while those in white colonies were white cells with smooth cell surface (Figs 3b and S2). WUM5A had a switching frequency of 84.5 ± 9.0% (Fig. 3c) while the switching frequency of the sac7Δ/Δ mutant was 14.0 ± 6.6% (Fig. 3a), indicating that the deletion of Sac7 in diploid C. albicans cells severely impaired the white-to-opaque switching. Re-introduction of a WT Sac7 gene back into the sac7Δ/Δ mutant increased the switching frequency to 43.5 ± 7.6% (Fig. 3a–c). Furthermore, overexpressing Wor1 in sac7Δ/Δ cells (sac7Δ/Δ + WOR1OE) from the Tet-off promoter completely suppressed the white-to-opaque switching defect. All the colonies of the sac7Δ/Δ + WOR1OE strain were stained red and contained only opaque cells (Fig. 3a–c). Similarly, the sac7Δ/Δ mutant was also defective in CO2-induced white-to-opaque switching, which could be largely rescued by re-introduction of a WT Sac7 gene.
The results provide further evidence that Sac7 has a role in the regulation of the white-to-opaque switching which is mediated by Wor1 in both haploid and diploid cells.

Overexpression of SAC7 enhances the white-to-opaque switching. Since the deletion of SAC7 reduced the white-to-opaque switching frequency, we expected that overexpression of SAC7 would promote the switching. To test this idea, a SAC7-overexpressing plasmid driven by the Tet-off promoter were introduced into WUM5A (+ SAC7OE) and the switching frequency were determined on Lee's GlcNAc plates (pH 6.0, +PB). After the induction, the WUM5A (+ SAC7OE) strain exhibited a switching frequency of 97.0 ± 1.5% which was significantly higher than the 83.4 ± 4.7% shown by the strain transformed with the empty vector (Fig. 4a,b,c). The data above indicate that Sac7 is a positive regulator of the white-to-opaque switching.

Sac7 physically interacts with Rho1. In Saccharomyces cerevisiae (Sc), Sac7 is known to be a GAP of Rho1 and physical interaction between them has been demonstrated. Next, we performed co-immunoprecipitation to determine whether Sac7 also interacts with Rho1 in C. albicans. Being aware of the cycling of Rho1 between active and inactive forms, we sought to construct constitutively active and inactive versions of Rho1. Sequence alignment of CaRho1 with the corresponding mutants of the Rho GTPase ScCdc42 guided us to generate two C. albicans strains expressing the Myc-Rho1G18V (GTP-locked) and Myc-Rho1D124A (GDP-locked) mutant GTPase, respectively. We constructed strains co-expressing Sac7-HA with Myc-Rho1G18V or Myc-Rho1D124A, immunoprecipitated Sac7-HA with a HA antibody from cell extracts, and probed the precipitation products using anti-Myc WB. We could only detect the interaction of Sac7 with Myc-Rho1G18V but not Myc-Rho1D124A (Fig. 5b). As the negative control, in cell extracts expressing Myc-Rho1G18V alone, we did not detect Rho1 in the anti-HA precipitation products, indicating that the detection of Myc-Rho1G18V was due to specific interaction with Sac7-HA (Fig. 5b). Together, the results demonstrate that Sac7 physically associates with the active, GTP-bound form of Rho1 in vivo.

Rho1-GTP negatively regulates the white-to-opaque switching. Above, we have provided evidence that Sac7 promotes the white-to-opaque transition. As Sac7 is a GAP of Rho1, we hypothesized that Rho1-GTP would negatively regulate the white-to-opaque switching. To test this hypothesis, we overexpressed, from the Tet-off promoter, the WT (RHO1WT), constitutively active (RHO1G18V) or constitutively inactive (RHO1D124A) RHO1 in WUM5A. The white-to-opaque switching frequency was determined by growing cells on Lee's GlcNAc plates (pH 6.0, +PB) in the dark at 25 °C for 6 days. The results showed that the switching frequencies of cells overexpressing RHO1WT, RHO1G18V and RHO1D124A were 89.4 ± 5.1%, 25.0 ± 8.2% and 99.8 ± 0.4%, respectively (Fig. 6a–c). A similar pattern was observed when these strains were subjected to CO2-induced white-to-opaque switching assay (Fig. S4). The data demonstrate that the active Rho1-GTP negatively regulates the white-to-opaque switching, which is consistent with the effect of SAC7 deletion and overexpression on the switching.
To investigate whether the regulation of the white-to-opaque switching by Rho1 is also mediated by Wor1, we overexpressed WOR1 in RHO1G18V cells. The switching assay showed that all the colonies of RHO1G18V+WOR1OE cells were stained red and contained only opaque cells (Fig. 6a,b) in sharp contrast to the 25.0 ± 8.2% switching frequency shown by the RHO1G18V cells (Fig. 6c). The data indicate that Rho1 functions upstream of Wor1.

\[ \text{ras1}\Delta/\Delta \text{exacerbates the white-to-opaque switching defect of sac7}\Delta/\Delta \text{mutant.} \]

A previous study has shown that the Ras1/cAMP signaling pathway plays an important role in the white-to-opaque switching of \textit{C. albicans} and acts upstream of Wor1. To explore the relationship between Rho1/Sac7 and the Ras1/cAMP pathway, we constructed a sac7\Delta/\Delta ras1\Delta/\Delta double mutant and tested its ability to undergo the white-to-opaque switching. The sac7\Delta/\Delta ras1\Delta/\Delta mutant displayed a significantly lower switching frequency than either one of the single mutants deleted of SAC7 or RAS1 alone (Fig. 7a,b). The switching frequencies were 3.1 ± 2.4% for sac7\Delta/\Delta ras1\Delta/\Delta, 14.5 ± 4.0% for sac7\Delta/\Delta, and 13.4 ± 2.5% for ras1\Delta/\Delta cells (Fig. 7c). The results suggest that Rho1/Sac7 function in a pathway in parallel with the Ras1/cAMP signaling cascade to regulate the white-to-opaque switching in \textit{C. albicans}.

**Discussion**

In this study, we have identified Sac7 as a new regulator of the white-to-opaque switching in \textit{C. albicans}. Sac7 is a GAP that negatively regulates the small GTPase Rho1. Our data indicate that Sac7 promotes the white-to-opaque switching by converting Rho1 from its GTP-bound active to GDP-bound inactive form. Epistatic analyses revealed that Rho1/Sac7 functions in a signaling pathway in parallel with the Ras1/cAMP cascade to regulate the phenotypic switching. Hence, our study has discovered a new signaling pathway that controls a unique and important trait in the life cycle of the fungal pathogen \textit{C. albicans}.

Before this study, the function of \textit{C. albicans} Sac7 was uncharacterized. Based on its closest homologue in \textit{S. cerevisiae}, the protein is inferred to act as a Rho1 GAP. Our experiments have provided compelling evidence supporting this role. By co-immunoprecipitation, we demonstrated the physical association of Sac7 with the active Rho1G18V but not the inactive Rho1D124A protein \textit{in vivo} (Fig. 5b). Also, the strain overexpressing SAC7, which drives Rho1 towards the Rho1-GDP form, exhibited an increased white-to-opaque switching frequency to a level similar to that of the constitutively inactive Rho1D124A mutant (Figs 4 and 6). Also, the sac7\Delta/\Delta mutant, in which Rho1 is kept in the active Rho1-GTP form, showed significantly reduced switching frequencies like the constitutively active Rho1G18V mutant (Figs 3 and 6). In \textit{S. cerevisiae}, Sac7 plays an important role in the organization
of actin cytoskeleton\(^43,44\) and cell wall organization\(^45,46\). We have observed that the \textit{Sac7}\(\Delta/\Delta\) diploid mutant of \textit{C. albicans} was sensitive to several cell wall toxins including SDS, Calcofluor White, and Congo Red (Fig. S5), consistent with a role for \textit{Sac7} in maintaining the cell wall integrity.

In \textit{S. cerevisiae}, \textit{Rho1} is an important regulator of diverse cellular functions including cell polarity, actin cytoskeleton organization, and cell wall biosynthesis\(^47\). It executes multiple tasks by binding and activating different effectors, such as \textit{Fks1} for cell wall remodeling\(^48–50\), \textit{Pck1} for cell integrity signaling\(^50–52\), \textit{Bem4} for bud emergence\(^53\), \textit{Bni1} for actin cytoskeleton reorganization\(^54\), and \textit{Sec3} for exocytosis\(^55\). In \textit{C. albicans}, \textit{Rho1} has been shown to interact directly with \textit{Gsc1} (also known as \textit{Fks1}), the \(\beta\)-1,3-glucan synthase catalytic subunit\(^56\) and regulates cell wall biosynthesis. Depletion of \textit{Rho1} resulted in cell death, lysis, and aggregation and the failure to colonize the kidney of mice during systemic infection\(^57\), indicating an essential role for \textit{Rho1} in cell viability and virulence. \textit{Rho1} is also required for \textit{C. albicans} invasive filamentous growth\(^38\). Recently, \textit{Rho1} was found to regulate morphogenesis via interaction with the GAP \textit{Lrg1}\(^58\). In addition to cell wall integrity and morphogenesis, whether \textit{Rho1} also regulates other cellular processes in \textit{C. albicans} remains to be determined. Our study expands \textit{Rho1}'s role to the regulation of the white-to-opaque switching in \textit{C. albicans}.

A diverse range of environmental signals is known to influence the white-to-opaque switching in \textit{C. albicans}\(^4\). However, our knowledge of the signaling pathways linking the external signals to the circuit of transcription factors that directly control the switching remains limited. The Ras/cAMP pathway has been demonstrated to mediate the white-to-opaque switching in response to GlcNAc and CO\(_2\)\(^26\). Our finding that \textit{Sac7}\(\Delta/\Delta\) and \textit{ras1}\(\Delta\) act synergistically to repress the switching (Fig. 7) suggests that \textit{Sac7}/\textit{Rho1} and \textit{Ras1} function in independent pathways. On the other hand, the suppression of the white-to-opaque switching deficiency of \textit{Sac7}\(\Delta/\Delta\) and \textit{rho1}\(G18V\) mutants by \textit{WOR1} overexpression (Figs 3 and 6) indicates that, like the Ras/cAMP pathway, \textit{Sac7}/\textit{Rho1} functions upstream of \textit{Wor1}. Here, we propose a working model to illustrate how \textit{Sac7}/\textit{Rho1} regulates the white-to-opaque switching (Fig. 8). Under non-inducing conditions, \textit{Sac7} is inactive and \textit{Rho1} is thus in the active \textit{Rho1}-GTP form. The active \textit{Rho1} then binds and activates its effector(s) which in turn activates downstream targets, leading to the inhibition of \textit{Wor1} and the white-to-opaque switching. Under inducing conditions, \textit{Sac7} is activated and \textit{Rho1}-GTP is converted to the inactive \textit{Rho1}-GDP form. Consequently, the \textit{Rho1} effector(s) becomes inactive and the inhibition on \textit{Wor1} is relieved, allowing cells to undergo the white-to-opaque switching. Currently, the identity of the effector(s) of \textit{Rho1} and the downstream signaling pathways linking to \textit{Wor1} remain unclear. One possibility is that \textit{Rho1}, like its counterpart in \textit{S. cerevisiae}, may interact with \textit{Pck1} to initiate the mitogen-activated

\textbf{Figure 6.} \textit{Rho1} negatively regulates the white-to-opaque switching. (a) Cells of WUM5A transformed with vector (YSL509), \textit{RHO1}\(WT\) (YSL617), \textit{RHO1}\(_{G18V}\) (YSL608), \textit{RHO1}\(_{D124A}\) (YSL613), or \textit{RHO1}\(_{G18V}+WOR1OE\) (YSL616) were grown on Lee's GlcNAC plates (pH 6.0, + PB) at 25°C in the dark for 6 days. Colonies formed by each strain were photographed and representative images are shown. (b) Representative images of white and opaque cells from the indicated strains. (c) The white-to-opaque switching frequency for WUM5A cells transformed with vector, \textit{RHO1}\(_{WT}\), \textit{RHO1}\(_{G18V}\), \textit{RHO1}\(_{D124A}\), or \textit{RHO1}\(_{G18V}+WOR1OE\). *\(p < 0.05\) (Student's \(t\)-test).
protein (MAP) kinase cascade, eventually leading to the phosphorylation and activation of the MAP kinase Mkc1 (the last member of the MAP cascade) which in turn directly or indirectly inhibits Wor1. In support of this model, we have observed that Mkc1 is hyper-phosphorylated in the \textit{sac7Δ/Δ} mutant and \textit{MKK2} (MAP kinase kinase) overexpressing cells. Furthermore, the white-to-opaque switching frequency of \textit{MKK2} overexpressing cells was reduced to a level similar to that of \textit{sac7Δ/Δ} (Fig. S6). However, the physical interaction between Rho1 and Pkc1 has not been demonstrated and whether Wor1 is phospho-regulated by Mkc1 remains to be determined, although Wor1 has been shown to be phosphorylated by protein kinase A (PKA) 26. Further investigations are required to conclude whether the Mkc1 MAP kinase cascade connects Rho1/Sac7 to Wor1 in the regulation of the white-to-opaque switching.

In this study, we have only tested three inducers lower pH, GlcNAc and CO\textsubscript{2} that promote the white-to-opaque switching via the Rho1/Sac7 signaling pathway. Many environmental cues, such as the sugar source, temperature, low doses of UV irradiation, genotoxic and oxidative stress, can influence the white-to-opaque switching and the stability of the opaque phenotype 4. Whether Sac7/Rho1 also has a role in mediating cellular responses to these signals remains to be determined.

We noticed that the white and opaque colonies formed by haploids and diploids have some differences. For example, haploid opaque colonies are slightly smaller than white colonies (Figs 1 and 2). In contrast, diploid opaque colonies are much bigger than white colonies (Fig. 3a). The reason causing such differences is unclear, but it is worthy to point out that haploids and diploids were induced to form opaque colonies by different inducers. Haploids were induced on YPD (pH 6.0) plates, while diploids were induced on Lee’s GlcNAc plates (pH 6.0). We have tried to induce haploid cells on Lee’s GlcNAc plates, but only tiny colonies could be formed and they were too small for accurate counting of white and opaque colonies. We also noticed that the diploid opaque cells have two types of morphology: oval-shaped or bean-shaped. The opaque cells of the wild-type diploid strain (WUM5A) were oval-shaped, whereas the opaque cells of \textit{sac7\OE} and \textit{RHO1\textsuperscript{G18V}} strains are bean-shaped. Interestingly, the opaque cells of WUM5A
changed their morphology from oval-shaped to bean-shaped after cultured in liquid Lee’s GlcNAc medium for three more days (Figs 3b and S2), suggesting that culture condition may play a role in determining the morphology of opaque cells. Although what causes the morphological change remains to be determined, it is evident that both oval- and bean-shaped cells were bona fide opaque cells, because they possessed the characteristics of opaque cells5,6, including bigger sizes, a pimpled surface, giant vacuoles, and staining by Phloxine B.

We have developed a more precise method than PB staining to distinguish opaque and white cells. We used the OP4 promoter to control the expression of C. albicans codon-optimized red florescent protein dTomato39. As OP4 is opaque-specific, the dTomato signal can only be detected in opaque cells. On the colony level, two types of dTomato signals could be observed. One is bright sectors containing only opaque cells, and the other has areas with punctate signals that contain both opaque and white cells. On the cellular level, the dTomato signal is distributed throughout the entire cytoplasm in opaque cells except for the vacuoles (Figs 1b and 2). In contrast, PB staining can only distinguish opaque cells from white cells on the colony level but not the cellular level. We found that PB stained not only opaque but also white colonies of diploid and haploid strains derived from SC5314 with ura3 auxotrophy, such as CAI4, BWP17 and GZY803, often leading to misidentification of opaque colonies. In comparison, dTomato driven by the OP4 promoter offers a convenient and reliable method to distinguish opaque and white cells.

C. albicans haploids were discovered recently to be derived from diploid parents via the concerted loss of one set of chromosomes30. Although the haploids did not show virulence in the mouse model of systemic infection, they retained key characteristics that define the species, including the yeast-to-hyphae transition, the white-to-opaque switching, mating, and the formation of chlamydospores30. The isolation of haploid C. albicans and subsequent construction of tool strains30,31 have opened up opportunities for performing genetic screens that had been very difficult to do in the diploids. We have constructed several small gene-deletion libraries based on gene functions, one of which includes genes encoding GTPases and their regulators. By screening this library, we have identified new regulators, GYP1 and IRA2, of hyphal growth and biofilm formation, two traits crucial for virulence. Importantly, the findings have been validated in C. albicans diploids32, demonstrating the enormous potential of conducting large-scale genetic screens to find new genes important for virulence as well as other biological processes. In this study, we have again presented a case of successfully identifying a new regulator of the white-to-opaque switching by first screening a haploid mutant library.

**Methods**

**Strains, plasmids, and growth conditions.** The C. albicans strains and plasmids used in this study are described in Table S1 and S2, respectively. All haploid strains were verified for ploidy by flow cytometry analysis according to the published protocol40. Recombinant DNA manipulations were performed according to standard methods. E. coli XL1 blue (Stratagene) was used as the host strain for recombinant plasmids and cultured in LB broth (0.5% yeast extract, 1% tryptone, and 0.5% NaCl, pH 7.0) supplemented with 100 μg/ml ampicillin. Site-directed mutagenesis followed the manual of the Quikchange multi-site-directed mutagenesis kit (Agilent Technologies). C. albicans cells were routinely grown at 30 °C in YPD (2% yeast extract, 1% peptone, and 2%
glucose), or GMM (glucose minimal medium, 6.79 g/l yeast nitrogen base without amino acids, and 2% glucose) supplemented with appropriate amino acids and other compounds (80 μg/ml uridine, 40 μg/ml arginine, 40 μg/ml histidine, and 1 mg/ml 5-Fluoroorotic acid) when necessary. Solid medium plates were prepared by adding 2% of agar. Lee’s medium containing N-acetylglucosamine as the carbon source (Lee’s GlcNAc) was prepared as previously described and the pH was adjusted to 6.0. Transformation of *C. albicans* with plasmids containing prototrophic markers was performed using the Fast Yeast Transformation Kit (G-Biosciences). The electroporation method was used to transform *C. albicans* with plasmids containing *SAT1* as the selection marker, and transformants were selected on YPD plates containing 200 μg/ml nourseothricin (Jena Biosciences). All gene deletions were verified by colony PCR as described, and looping out of *URA3* via FLP-mediated excision followed the previous protocol.

**White-to-opaque switching assays.** To induce the white-to-opaque switching in haploid strains, cells were first grown on YPD plates at 25 °C for 1–2 days, then diluted with water and plated onto YPD plates (pH 6.0) containing 5 μg/ml of PB. The plates were incubated in the dark at 25 °C for 7 days to allow the development of single colonies (around 50–80 colonies per plate). The plates were then photographed with a digital camera and the numbers of colonies exhibiting different color patterns were counted. Cells from representative colonies were picked and suspended into water for morphological examination under an optical microscope (Leica LEITZ DM RXZ) equipped with a Moticam 10 digital camera. Images were acquired with the MetaMorph 7.5 software (MDS Analytical Technologies).

**Imaging of dTomato signals.** Haploid strains transformed with dTomato-expressing plasmid were first grown on YPD plates at 25 °C for 3–4 days, then diluted with water and plated onto Lee’s GlcNAc plates (pH 6.0) containing 5 μg/ml of PB. The plates were incubated in the dark at 25 °C for 6 days to allow the development of single colonies (around 100–150 colonies per plate) and then photographed. The colonies exhibiting different color patterns were counted. Switching frequency was calculated as the percentage of red colonies and white colonies with red sectors over the number of total colonies. Cells from representative colonies were picked and suspended into water for morphological examination under Leica DMRX-A2 microscope equipped with a CoolSnap HQ2 digital camera (Roper Scientific). Differential interference contrast (DIC) images were acquired using the MetaMorph 7.5 software (MDS Analytical Technologies).

**Protein extraction, immunoprecipitation (IP), and Western blotting (WB).** To prepare yeast lysates, cells were harvested into 2 ml screw-cap microcentrifuge tubes by brief centrifugation to obtain pellets with a volume ≤ 500 μl and resuspended in 500 μl of ice-cold yeast lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM KCl, 1% NP-40) containing the protease inhibitor cocktail (Nacalai Tesque Inc). After adding an equal volume of acid-washed glass beads (Sigma-Aldrich), cells were broken by 6 rounds of 60-second beating at 5000 rpm in a MicroSmash MS–100 beater (TOMY Medico) with 1 min of cooling on ice between rounds. The lysed cells were then centrifuged at 16,000 rpm for 15 min at 4 °C and supernatants were collected.

To perform IP, yeast lysate was incubated with 30 μl of the slurry of rabbit polyclonal HA or Myc beads (Santa Cruz Biotechnology) at 4 °C for 1 h. After brief centrifugation at 800 rpm, the beads were washed 3 times by resuspension in 1 ml cold yeast lysis buffer followed by brief centrifugation. The washed beads were resuspended in 20 μl 2x protein loading buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 200 mM dithiothreitol, 0.02% bromophenol blue) and boiled for 10 min. Protein samples were separated by 10% SDS-PAGE gel and subsequently transferred to a PVDF membrane (Bio-Rad Laboratories).

For WB analysis, the PVDF membrane was first incubated with 5% milk blocking solution (dissolved in PBS containing 0.1% Tween-20, PBST) at room temperature for 1 h or at 4 °C overnight. After a brief rinse with PBST, the membrane was incubated with PBST containing a 1:1000 diluted primary antibody (mouse monoclonal HA or mouse monoclonal Myc antibody from Nacalai Tesque Inc) at room temperature for 1 h, followed by 3 rounds of 5-min wash with PBST. The membrane was then incubated with PBST containing a 1:5000 diluted secondary antibody (HRP-linked anti-mouse IgG from sheep; GE Healthcare). After 3 rounds of 5-min wash with PBST, the membrane was immersed in Pierce ECL WB substrate solution (Thermo Scientific) and exposed to X-film.

**Statistical analysis.** The white-to-opaque switching frequency was calculated as the percentage (with standard deviation) of red/pink-containing colonies (red/pink colonies and white colonies with red/pink regions) over total colonies. Statistical analyses were performed using two-tailed, unpaired Student’s t-tests in Excel. p values < 0.05 were considered significant. All experiments were carried out at least in triplicate.

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Author Contributions
S.L.Y., conceptualization of the project, conducting experiments and data analysis; G.S.Z., conceptualization of the project, conducting experiments and data analysis, preparation of manuscript; Y.M.W., F.Y.C., and Y.D.L., conducting experiments; Y.W., conceptualization and supervision of the project, preparation of the manuscript.

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