KRE5 Suppression Induces Cell Wall Stress and Alternative ER Stress Response Required for Maintaining Cell Wall Integrity in Candida glabrata

Yutaka Tanaka¹, Masato Sasaki¹, Fumie Ito¹, Toshio Aoyama², Michiyo Sato-Okamoto³, Azusa Takahashi-Nakaguchi³, Hiroji Chibana³, Nobuyuki Shibata¹*

¹ Department of Infection and Host Defense, Tohoku Medical and Pharmaceutical University, Sendai, Japan, ² Department of Electronic and Information Engineering, Suzuka National College of Technology, Suzuka, Japan, ³ Medical Mycology Research Center, Chiba University, Chiba, Japan

* nshibata@tohoku-mpu.ac.jp

Abstract

The maintenance of cell wall integrity in fungi is required for normal cell growth, division, hyphae formation, and antifungal tolerance. We observed that endoplasmic reticulum stress regulated cell wall integrity in Candida glabrata, which possesses uniquely evolved mechanisms for unfolded protein response mechanisms. Tetracycline-mediated suppression of KRE5, which encodes a predicted UDP-glucose:glycoprotein glucosyltransferase localized in the endoplasmic reticulum, significantly increased cell wall chitin content and decreased cell wall β-1,6-glucan content. KRE5 repression induced endoplasmic reticulum stress-related gene expression and MAP kinase pathway activation, including Slt2p and Hog1p phosphorylation, through the cell wall integrity signaling pathway. Moreover, the calcineurin pathway negatively regulated cell wall integrity, but not the reduction of β-1,6-glucan content. These results indicate that KRE5 is required for maintaining both endoplasmic reticulum homeostasis and cell wall integrity, and that the calcineurin pathway acts as a regulator of chitin-glucan balance in the cell wall and as an alternative mediator of endoplasmic reticulum stress in C. glabrata.

Introduction

Maintenance of cell wall integrity (CWI) is critical in fungal biology [1–6]. The fungal cell wall is required for normal cell growth and acts as a physical support for maintaining fungal cell shape and as a barrier for protection against harsh environments [7,8]. Changing environmental conditions induce various signal transduction pathways that contribute to remodeling of cell wall physiology [9]. Of these pathways, the CWI pathway depends on a signal transduction mechanism involving the MAP kinase cascade, whose triggers are directly involved in cell wall remodeling [10,11]. Some in vitro studies have shown that up-regulation of the CWI pathway in the pathogenic yeast Candida glabrata induce resistance to echinocandin antifungal drugs at
clinically relevant supra-minimum inhibitory concentrations (MIC) [12–14]. It is also well known that lack of cell wall β-1,6-glucan causes severe growth defects and strongly induces CWI [15]. The cell wall of C. glabrata is composed of mannoproteins, β-1,3-glycans, β-1,6-glycans, and chitin [16]. Cell wall metabolism in C. glabrata has been characterized by comparative genomic analyses of Saccharomyces cerevisiae [17–19], and β-1,6-glycans in C. glabrata act as a linker between mannoproteins and chitin in the outer cell wall across the cell wall structure [15,20]. These findings suggest that β-1,6-glycans play an important role in maintaining a certain cell wall structure, and disruption of CWI is expected to be a new target for antifungal drugs.

A recent study showed that endoplasmic reticulum (ER) homeostasis is required for maintaining proper cell wall structure and for inducing antifungal resistance in many fungal species such as Saccharomyces cerevisiae [21], Aspergillus fumigatus [22], and Cryptococcus neoformans [23]. Unfolded protein response (UPR) is a well-conserved reaction in most eukaryotes for maintaining ER homeostasis [24,25]. Saccharomyces cerevisiae has a canonical UPR signaling system, the IRE1-HAC1 pathway, whereas humans have two other UPR pathways [26–29]. C. glabrata lacks the canonical IRE1-HAC1 pathway for the UPR, which is required for transmitting ER stress accumulation signals to the cytoplasm [30]. Nonetheless, C. glabrata has primary resistance against a typical ER stress inducer, tunicamycin (TM), and treatment with TM induces the expression of several genes required for maintaining the proper cell wall structure [31]. This suggests that C. glabrata has different UPR mechanisms regulating the CWI pathway.

KRE5, which belongs to the KRE family of genes, is predicted to be involved in cell wall β-1,6-glucan synthesis in many eukaryotes, including Saccharomyces cerevisiae [32] and Candida albicans [33]. KRE5 encodes a soluble luminal ER protein containing a highly conserved UDP-glucose glycoprotein:glucosyltransferase (UGGT) domain in its C-terminus. In several eukaryotes, the UGGT domain is involved in the folding of nascent secretory proteins, including some cell wall component synthases, in the ER with the help of ER chaperones such as calnexin [34–36]. Deletion of the gene encoding UGGT induces dramatic cell wall alterations in many fungi [32,33,37]; however, the UGGT domain of S. cerevisiae Kre5p does not function as a co-chaperone of calnexin, in contrast to the Kre5p of other fungi [8]. Although C. glabrata is phylogenetically similar to S. cerevisiae [38], the function of C. glabrata Kre5p is unclear. Mutations in other KRE family genes result in a viable phenotype in most cases; however, mutations in C. glabrata KRE5 induce a lethal phenotype described later in this study. Therefore, we hypothesized that KRE5 has an epistatic function affecting the growth and CWI in C. glabrata. In the present study, we characterized the functions of C. glabrata KRE5 by generating a KRE5 mutant with a regulatable gene expression system, and determined whether ER-mediated CWI was induced by the repression of ER-localized Kre5p.

Results

C. glabrata possesses a single gene similar to Saccharomyces cerevisiae KRE5

We identified an uncharacterized gene, CAGL0E05412g (http://www.genolevures.org/), that was highly similar to Saccharomyces cerevisiae KRE5 (S1 Fig). CAGL0E05412g encoded a protein containing 1,326 amino acids, with a predicted molecular weight of 152.3 kDa and an ER translocation signaling sequence at its N-terminus. The C-terminus of the predicted protein contained a domain that was significantly similar to the UGGT domain, which is highly conserved in many eukaryotes and in the Kre5p of fungi belonging to the phyla Ascomycota. Therefore, the CAGL0E05412g gene was regarded as a functional homologue of KRE5, and was designated as C. glabrata KRE5 (CgKRE5).
CgKRE5 is indispensable for cell survival

To investigate the role of CgKRE5, we replaced the CgKRE5 ORF with a selectable marker; however, we could not generate a CgKRE5 disruption mutant (data not shown). As reported previously, almost all haploid KRE5-null mutants of Saccharomyces cerevisiae strains are non-viable. Therefore, we predicted that CgKRE5 disruption induced a lethal phenotype in C. glabrata. Then, we used a tetracycline-dependent system to repress CgKRE5 (Fig 1A). A tetracycline-dependent down-regulatable promoter (tet-off promoter) [39] was inserted upstream of the CgKRE5 ORF in the parent C. glabrata HETS202 strain, and the resulting strains were genotyped by Southern blot analysis to confirm the correct integration site (Fig 1B). The tet-off strain showed significant reduction in CgKRE5 mRNA expression in the presence of 20 μg mL⁻¹ doxycycline (DOX) (Fig 1C). Because mutations in KRE family genes induce a killer toxin-resistant phenotype in Saccharomyces cerevisiae [40], we performed an inhibition ring test to determine whether CgKRE5 repression induced a killer toxin-resistant phenotype. We observed that DOX-treated cells incubated with K-1 killer toxin formed a small inhibition ring (Fig 1D). Furthermore, CgKRE5 repression could be complemented by expression of Saccharomyces cerevisiae Kre5p (S2 Fig). Thus, we successfully generated a tet-regulatable KRE5 strain with DOX-dependent CgKRE5 expression, and CgKRE5 was characterized as a KRE family gene even in C. glabrata.

CgKRE5 is involved in cell wall morphogenesis

Because KRE family genes are predicted to be involved in cell wall synthesis, we measured the major cell wall components, β-glucan and chitin, in the CgKRE5 repression mutant. DOX-treated tet-KRE5 cells showed approximately 50% reduction in β-1,6-glucan content (Fig 2A) but no significant reduction in β-1,3-glucan content (Fig 2B). This result indicates that CgKRE5 plays an important role in maintaining cell wall β-1,6-glucan content. Furthermore, cell wall chitin content was considerably increased in DOX-treated tet-KRE5 cells (Fig 2C). These data suggest that CgKRE5 repression induced cell wall decomposition, which is characterized by a decrease in cell wall β-1,6-glucan content and an abnormal accumulation of cell wall chitin content. We next investigated whether CgKRE5 repression affected not only cell wall structure but also cell wall homeostasis by effects on cell wall effector molecules that induce cell wall stress [41–43]. Compared to HETS202 cells and DOX-untreated cells, DOX-treated tet-KRE5 cells showed a decreased rate of cell growth (Fig 2D). Importantly, DOX-treated tet-KRE5 cells exhibited hypersensitivity to Congo red (CR) and calcofluor white (CFW), which covalently bind to cell wall β-1,3-glucans and chitin, respectively (Fig 2D). These data imply that the DOX-treated tet-KRE5 cells had a fragile cell wall because of the decrease in cell wall β-1,6-glucan content, and could not bear further stress. Moreover, these results indicate that appropriate CgKRE5 expression was required for maintaining normal cell wall physiology, and that CgKRE5 repression induced abnormalities in cell wall catabolism through the CWI pathway.

CgSLT2 mediates CgKRE5 repression-induced cell wall chitin synthesis, and its deletion leads to further suppression of vegetative growth

To determine the impact of these cell wall defects on cell viability, we evaluated important properties such as growth rate, cell shape, and cell cycle. DOX-treated tet-KRE5 cells showed significant growth defects in liquid YPD medium (Fig 3A). This result indicates that CgKRE5 is required for normal growth of C. glabrata. Microscopic analysis revealed that tet-KRE5 cells were highly agglutinated, with an irregular shape and size (Fig 3B). Furthermore, the number
Fig 1. Generation of tet-KRE5 cells. (A) Schematic representation of the integration of the controllable tet-off promoter. Region A represents the 5’ flanking region of the target gene and region B represents the 5’ end of the ORF. DNA fragment containing the controllable cassette in which CgHIS3 and tetO-ScHOP1 promoter were flanked by regions A and B, was used to transform C. glabrata HETS202 cells. (B) Correct integration of the tet-off promoter in the CgKRE5 promoter locus was confirmed by Southern blot analysis. Genomic DNA isolated from both transformants and HETS202 cells was digested with NdeI, and hybridized by CgKRE5 internal DNA probe. (C) Relative CgKRE5 expression was determined by performing RT-PCR. Data are expressed as the mean fold difference between HETS202 and tet-KRE5 cells treated with or without DOX. The value of HETS202 cells without DOX treatment was set as 1. Results are expressed as the mean standard deviation (S.D.) of triplicates; *, p < 0.05. (D) Inhibition ring test with K-1 killer toxin. C. glabrata cells were streaked on YPD solid medium, and 1 mg mL⁻¹ of K-1 killer toxin solution was spotted onto paper discs that were then placed on the YPD solid medium inoculated with C. glabrata.
doi:10.1371/journal.pone.0161371.g001
of DOX-treated tet-KRE5 cells in the G2/M phase was lower than that of DOX-untreated cells (Fig 3C). In addition, the number of aneuploid cells in the DOX-treated tet-KRE5 cells increased. These results suggest that CgKRE5 repression affected normal cell growth, which in turn exacerbated morphogenesis checkpoint defects and prevented proper bud formation as a result of the cell wall β-glucan-chitin imbalance.

Because up-regulation of chitin in the cell wall has been often observed in extrinsic cell wall damage, we considered that CgKRE5 repression might activate CWI activation to compensate for the lethal cell wall defect. In the event of a cell wall defect, the intracellular SLT2 and HOG1 MAP kinase cascade is activated to maintain the cell wall structure [42,44]. Because Slt2p phosphorylation is required for the activation of the CWI pathway [45,46], we monitored the

![Fig 2. CgKRE5 is involved in cell wall morphogenesis. Alkali-insoluble β-1,6-glucan (A), β-1,3-glucan (B), and chitin (C) contents in C. glabrata cells were measured as the quantity of glucose or glucosamine substituted for the standard curve. Data shown represent the results of at least three independent experiments. Error bars represent standard deviations; *, p < 0.05. (D) Spot dilution assay was performed using 5 μL suspensions at an OD of 0.1, and serially diluted (1:5) cells were spotted on yeast extract-peptone-dextrose (YPD) plates with or without DOX and were incubated at 37°C. A representative image of three independent experiments is shown.](image-url)
KRE5 is Required for Maintaining CWI and ER Homeostasis

(A) HETS202

(B) DOX (-) DOX (+)

(C) HETS202 tet-KRE5

Cell count

DNA content

G_{i} S G_{s/M} aneuploid

0 50 100 150 200

0 6 12 24 48

log (OD_{opt})

Time (h)

log (OD_{opt})

Time (h)

DOX (-) DOX (+)

HETS202 tet-KRE5
phosphorylation of both Slt2p and Hog1p to determine whether CgKRE5 repression causes CWI activation. Consistent with the cell wall alteration in KRE5 repression, both Slt2p and Hog1p were significantly phosphorylated in DOX-treated tet-KRE5 cells, suggesting that CgKRE5 repression induces CWI pathway activation (Fig 4A). To determine whether Slt2p plays a role in cell wall chitin complementation in CgKRE5 repression, we constructed a CgSLT2 deletion mutant from the tet-KRE5 strain. Combined disruption of CgKRE5 and CgSLT2 completely suppressed up-regulation of cell wall chitin (Fig 4B), but did not exert any effect on β-1,6-glucan content (Fig 4C). These data indicate that CgKRE5 directly regulates β-1,6-glucan synthesis and indirectly induces chitin synthesis through the Slt2p pathway. Furthermore, CgSLT2 deletion in the presence of CgKRE5 repression significantly suppressed vegetative growth (Fig 4D, upper). These results suggest that Slt2-mediated CWI plays a crucial role in maintaining the cell wall under CgKRE5 repression.

CgKRE5 repression induces ER stress

Next, we considered how C. glabrata senses cell wall abnormalities and transmits the signal for activating CWI by repressing the ER protein Kre5p. Treatment with the typical ER stress inducer TM resulted in phosphorylation of both Slt2p and Hog1p (Fig 4A). TM treatment increased cell wall chitin content in both HETS202 and tet-KRE5 cells (Fig 4B), but did not affect β-1,6-glucan content (Fig 4C). Moreover, CgSLT2 deletion in the presence of CgKRE5 repression significantly suppressed growth with TM treatment (Fig 4D, middle). These data suggest that ER stress also activated Slt2p-mediated CWI in C. glabrata. In addition, we observed that the growth of TM-treated cells was comparable to that of the parental and DOX-treated cells (Fig 5A). These results led us to hypothesize that CgKRE5 repression causes ER stress accumulation, and that ER stress induces CWI as UPR to dissolve structural abnormalities in the cell wall of C. glabrata. To confirm this, we initially analyzed the ER stress response after CgKRE5 repression. Co-treatment of DOX-treated tet-KRE5 cells with a calcineurin inhibitor FK-506 (tacrolimus) and Ca2+ chelator EGTA inhibited cell growth considerably (Fig 5A). Previous studies have suggested that the calcineurin pathway functions as the sole UPR pathway in C. glabrata, and that endogenous Ca2+ acts as an ER stress messenger [21,30,47]. Indeed, we observed that FK-506 treatment caused an extensive decrease of cell growth in DOX-treated tet-KRE5 and tet-KRE5ΔSLT2 cells (Fig 4D, bottom). Consistently, our results suggest that CgKRE5 repression induced hypersensitivity to exogenous ER stress induction and/or an imbalance in ER homeostasis, leading to the accumulation of endogenous basal ER stress. Moreover, recent studies have indicated that deletion of the ER stress-related protein, Ire1p, activates the CWI pathway. Therefore, we believe that CgKRE5 repression induces other cellular reactions, such ER stress and/or UPR, which lead to abnormal cell growth. To further determine whether CgKRE5 repression induced ER stress in C. glabrata, we examined the mRNA expression levels of representative UPR target genes, including KAR2 (a resident ER chaperone), BAG7 (a putative GTPase-activating protein involved in cell wall and cytoskeleton homeostasis), and YPS1 (involved in ER protein trafficking) via real-time PCR. Expression of these UPR target genes increased in DOX-treated tet-KRE5 cells (Fig 5B) and in TM-treated
Fig 4. CgKRE5 repression increased cell wall chitin content by activating the CWI-regulating MAP kinase pathway. (A) Phosphorylation of Slt2p and Hog1p was determined by performing western blotting analysis with antibodies against phosphorylated Slt2p and Hog1p (p-Slt2p and p-Hog1p, respectively). Anti-Pgk1p antibody was used as a loading control. Representative data of three independent experiments are shown; TM, tunicamycin. (B and C) Alkali-insoluble chitin (B) and β-1,6-glucan (C) content in C. glabrata cells were measured as the quality of glucose or glucosamine substituted for the standard curve. Data shown
CgKRE5 repression induced up-regulation of cell wall chitin content is further enhanced by treatment with the calcineurin inhibitor FK-506

As mentioned previously, CgKRE5 repression induced both cell wall decomposition and ER stress by phosphorylating Slt2p MAP kinase in C. glabrata. To investigate whether inhibition of CgKRE5 repression-induced ER stress affected cell wall structure, we co-treated tet-KRE5 cells with DOX and FK-506. Co-treatment of tet-KRE5 cells with DOX and FK-506 increased the transcriptional activation of CHS1 and GFA1 compared with that in tet-KRE5 cells treated with DOX alone (Fig 6A). CHS1 encodes chitin synthase, which is required for forming primary chitin septal plate [48], and GFA1 encodes glutamine:fructose-6-phosphate amidotransferase, which is required for the synthesizing metabolic precursors of cell wall chitin [49]. Increased CHS1 and GFA1 mRNA expression in tet-KRE5 cells co-treated with DOX and FK-506 increased cell wall chitin content (Fig 6B), but did not significantly affect cell wall β-1,6-glucan content (Fig 6C). Similarly, Miyazaki et al. reported that TM treatment induced CHS1 and GFA1 mRNA expression in C. glabrata [47], suggesting that CgKRE5 repression induced cell wall chitin content by activating transcription of CHS1 and GFA1 as an ER stress response. Moreover, it was suggested that the calcineurin pathway negatively regulated cell wall chitin synthesis.

To determine whether calcineurin regulated the Slt2p MAP kinase pathway, we analyzed Slt2p phosphorylation in FK-506-treated tet-KRE5 cells. Co-treatment of tet-KRE5 cells with FK-506 and DOX increased phosphorylation for both Slt2p and the Hog1p, whereas treatment of tet-KRE5 cells with only FK-506 did not affect the phosphorylation of either Slt2p or Hog1p (Fig 6D). Moreover, CgSLT2 deletion did not rescue the vegetative growth of FK-506-treated cells (Fig 4D). These data imply that ER stress activates the calcineurin pathway and partly regulates CWI driven by Slt2p activation. Remarkably, FK-506 treatment rescued cell aggregation induced by CgKRE5 repression but resulted in an increased number of ruptured cells (Fig 6E). These data suggest that inhibition of the calcineurin pathway induces cell growth defects under cell wall stress, and that the calcineurin complex plays an important role in negatively regulating the Slt2p pathway in CWI in C. glabrata.

CgKRE5 repression enhances the sensitivity of C. glabrata to micafungin

The integrity of cell surface components, including the cell wall and cell membrane, affects the efficacy of antifungal drugs. Recent studies have shown that increased cell wall chitin content thorough activation of the Slt2p induces resistance to echinocandin drugs in some Candida spp., including C. glabrata. Therefore, we examined the sensitivity of tet-KRE5 cells to micafungin and fluconazole. No significant difference in fluconazole sensitivity was observed after DOX treatment; however, DOX-treated tet-KRE5 cells showed modestly impaired growth in the presence of micafungin compared to the DOX-untreated cells (Fig 7). This result indicates that CgKRE5 repression enhances the sensitivity of C. glabrata to micafungin, despite the increase in cell wall chitin content.
Fig 5. Repression of CgKRE5 induces ER stress in C. glabrata. (A) Spot dilution assay was performed using the ER stress indicator. Five-microliter suspensions at an OD of 0.1 and serially diluted (1:5) cells were spotted on YPD plates with the indicated concentration of reagents incubated at 37°C. A representative image of three independent experiments is shown. (B) Real-time RT-PCR was performed to measure the mRNA expression levels of ER stress-associated genes. Amplification efficiencies were validated and were normalized using that of PGK1. Relative mRNA levels were calculated as the ratio of normalized mRNA level to the mRNA level of PGK1. Values are represented as the average of three independent experiments, and error bars indicate S.D.; *, p < 0.05 compared with DMSO-treated tet-KRE5 cells (Student’s t-test).

doi:10.1371/journal.pone.0161371.g005
Fig 6. Calcineurin inhibitor FK-506 upregulated cell wall chitin synthesis by accelerating Slt2p phosphorylation. (A) Relative mRNA levels of CHS1 and GFA1 were calculated from the ratio of the signal intensities of GFA1 and CHS1 mRNAs compared with that of PGK1 mRNA, which was used as a reference. Values are represented as an average of three independent experiments, and error bars indicate S.D.; *, p < 0.05, compared with DOX-untreated tet-KRE5 cells (Student’s t-test). (B and C) Cell wall composition of C. glabrata strains is shown. Alkali-insoluble chitin (B) and β-1,6-glucan (C) are quantified by the weight percent of the dry cell weight. (D) Western blot analysis of phosphorylated Slt2p, Hog1p, and Pgk1p in HETS202 and tet-KRE5 cells treated with DOX and/or FK-506. (E) Colony morphology of C. glabrata cells grown in medium containing DMSO or DOX with or without FK-506.
Discussion

*CgKRE5* plays an important role in the biosynthesis of β-1,6-glucan and maintenance of ER homeostasis

Cell wall β-1,6-glucan biosynthesis is one of the most intriguing processes in fungal cell biology. The *KRE* family gene products are localized in the ER-to-Golgi secretory pathway and are believed to be a key component for cell wall β-1,6-glucan synthesis [50]. Although it is still unclear which enzyme directly catalyzes this process and where the β-1,6-glucan is synthesized, our data clearly indicate that repression of *CgKRE5* induced ER stress accumulation and dynamic cell wall decomposition, with a higher reduction in cell wall β-1,6-glucan content. *CgKRE5* dysfunction induces cell wall stress by impairing the balance of cell wall β-glucan content and increased basal ER stress levels. In many eukaryotes, glycoprotein synthesis through the ER-to-Golgi secretory pathway is globally down-regulated under excessive ER stress accumulation [51]. The ER-to-Golgi secretory pathway promotes β-1,6-glucan synthesis, suggesting that important glycoproteins involved in β-1,6-glucan synthesis are down-regulated under ER stress after *CgKRE5* repression. Kurita *et al*. reported that proper folding and localization of Kre6p and Knh1p and of Cwh41p and Rot2p, which are localized in the ER and which act as co-chaperones of calnexin (Cne1p), are required for β-1,6-glucan synthesis [52]. In addition, a recent study showed that loss of the UPR function induces cell wall defects and that cell wall stress activates the UPR through the MAP kinase signaling pathway regulating CWI [53].

![Fig 7](https://doi.org/10.1371/journal.pone.0161371.g007)

**Fig 7.** *CgKRE5* repression enhances the sensitivity of *C. glabrata* to micafungin. Inhibition ring test by using micafungin or fluconazole is shown. *C. glabrata* cells were streaked on YPD solid medium, and indicated concentrations of micafungin or fluconazole were spotted onto paper discs, which were then placed on the YPD solid medium inoculated with *C. glabrata*.

1, 6-glucan (C) contents in *C. glabrata* cells were measured as the quantity of glucosamine or glucose substituted for the standard curve. Data shown represent the results of at least three independent experiments. Error bars represent standard deviations; *, p < 0.05; n.s, not significant. (D) Phosphorylation of Slt2p and Hog1p was determined by performing western blotting analysis with antibodies against p-Slt2p and p-Hog1p, respectively. Anti-Pgk1p antibody was used as the loading control. Representative data are shown of independent experiments is shown. (E) Microscopic analysis of tet-KRE5 cells. A representative image of three independent experiments is shown; scale bar: 10 μm; arrow, disrupted C. glabrata cells.
Consistent with these results, we found that loss of ER homeostasis induced defects in cell wall β-1,6-glucan biosynthesis and activated the CWI pathway.

However, the actual functions of CgKRE5 are still unclear. Schizosaccharomyces pombe gpt1 encodes a protein containing the UGGT domain [37] with an α-1,3-glucosyl transferase activity that adds a glucose residue to the terminal mannose of an immature N-linked glycan in the ER. In addition, a recent study showed that Gpt1p is involved in the UPR along with Ire1p, a luminal sensor of misfolded ER proteins in Schizosaccharomyces pombe [54] However, Saccharomyces cerevisiae Kre5p does not show such activity [8]. C. glabrata Kre5p contained the UGGT domain and a predicted ER retention signaling sequence at its C-terminus. We observed that CgKRE5 repression induced ER stress accumulation, which indicates that CgKRE5 is required for maintaining ER homeostasis in C. glabrata, similar to Schizosaccharomyces pombe gpt1.

Increased cell wall chitin content does not induce resistance to echinocandin drugs

Echinocandins such as micafungin and caspofungin inhibit the synthesis of cell wall β-glucans by competitively inhibiting β-1,3-glucan synthase Fks1p in C. glabrata. Previous studies have suggested that echinocandin treatment of most Candida spp. at supra-MIC induces paradoxical growth improvement by increasing cell wall chitin content and SLT2 mRNA expression [55–58]. In contrast, our results suggest that CgKRE5 repression induced micafungin hypersensitivity, despite activation of cell wall chitin synthesis and increased cell wall chitin content in C. glabrata. Echinocandin antifungal drugs inhibited β-1,3-glucan synthesis, and CgKRE5 repression decreased β-1,6-glucan content, implying that compensatory activation of chitin synthesis does not reverse excessive β-1,6-glucan synthesis in C. glabrata, and possibly in other fungi, similar to that observed with the CWI pathway. Although β-1,6-glucan content is much lower than β-1,3-glucan content in the cell walls of Candida spp., β-1,6-glucan chains play a pivotal role in the assembly of cell wall components [59,60]. The results of previous studies and those of the present study suggest that resistance to echinocandin antifungal drugs is not induced by an increase in cell wall chitin content. Overall, it is clear from our findings that inhibition of the β-1,6-glucan synthesis is a promising antifungal target.

Calcineurin inhibitor FK-506 disrupts fungal CWI, indicating its use as a potential antifungal drug

Calcineurin, a Ca²⁺/calmodulin-dependent phosphatase complex, has been investigated in detail mainly in S. cerevisiae and in mammalian cells. Previous studies have demonstrated that the calcineurin pathway plays pivotal roles in C. glabrata biology, such as in cell wall maintenance, antifungal susceptibility, and virulence induction in mice and other eukaryotes [61,62]. Recently, Miyazaki et al. reported that TM-induced ER stress results in the transcriptional activation of genes involved in chitin synthesis and that calcineurin and the Slt2p MAP kinase cascade play a uniquely important role in the ER stress response in C. glabrata, rather than the canonical UPR mechanism, i.e., the IRE1-HAC1 pathway [31]. We observed that CgKRE5 repression induced ER stress accumulation, Slt2p phosphorylation, and CHS1 and GFA1 mRNA up-regulation, thus increasing cell wall chitin content. Furthermore, inhibition of the calcineurin pathway activated the CWI pathway of cell wall remodeling and induced greater impairment of fungal cell growth. These findings indicate that the calcineurin pathway negatively regulates cell wall chitin content similarly to the CWI pathway under cell wall stress. In many fungi, the balance between cell wall chitin and β-glucan content is essential for normal
cell growth. Therefore, calcineurin plays an important role in maintaining CWI by negatively regulating ER stress-mediated chitin synthesis.

We observed that the calcineurin inhibitor FK-506 effectively disrupted the cell wall β-glucan-chitin balance under cell wall stress conditions. The defect in β-1,6-glucan synthesis induced by CgKRE5 repression was not dependent on the ER stress response, at least in the calcineurin pathway. A recent in vitro study showed that co-treatment of C. glabrata with tacrolimus and azole antifungal drugs exerted a strong antifungal effect [63], suggesting that inhibition of the calcineurin pathway induces severe growth defects in C. glabrata under cell wall stress, which was consistent with the findings of the present study. Patients with acute graft-versus-host disease (GvHD), a life-threatening immunological complication that occurs after hematopoietic stem cell transplantation, are treated with tacrolimus (FK-506) or cyclosporine A [64,65]. These immunosuppressive drugs are currently administered as prophylactic agents not only for preventing GvHD but also for preventing fungal infection after solid-organ transplantation [66,67]. Consistent with the application in these clinical therapies, our results suggest that FK-506 induced an imbalance in cell wall β-glucan and chitin content, thus inducing cell growth defects in C. glabrata, and that it exerted potential antifungal effects by disrupting fungal CWI.

Previous studies reported synergic effects of tacrolimus and azole antifungal agents on inhibition of the synthesis of fungal-type cell membranes [68,69]. In our experiment, fluconazole, a representative azole antifungal drug, did not exert antifungal activity under repression of KRE5. This result indicates that KRE5 repression-induced CWI maintained not only the cell wall but also the cell membrane, i.e., the cell “surface”. Therefore, co-treatment with tacrolimus and an azole antifungal drug might be more efficient against C. glabrata infection because tacrolimus would function as a CWI disruptor under cell wall stress conditions.

Concluding remarks

We clarified that CgKRE5 regulated CWI in C. glabrata. Our results also suggest that CgKRE5 plays a pivotal role in the maintenance of CWI because CgKRE5 dysfunction induced ER stress and activated the CWI pathway, which led us to propose a hypothetical model (Fig 8). During normal cell growth, delivery of cell wall precursors through the intracellular ER-to-Golgi secretory pathway maintains an appropriate cell wall structure. In the ER, Kre5p serves as a chaperone cycle member for proper folding of N-linked glycoproteins, which is a probable step in the synthesis of cell wall β-1,6-glucan (Fig 8A). CgKRE5 repression considerably decreases cell wall β-1,6-glucan content and the reciprocal increases cell wall chitin content by activating the CWI pathway. At the same time, Kre5p repression in C. glabrata induces ER stress and also the sole UPR in C. glabrata, the calcineurin pathway. Thus, CWI is maintained by maintenance of an appropriate chitin content by the calcineurin complex through an “intracellular check” (Fig 8B). FK-506 prevents the formation of the calcineurin complex, thus repressing the negative regulation of the CWI pathway. Increased cell wall chitin content resulting from dysfunction of the calcineurin pathway induces an imbalance in cell wall structure and affects normal cell growth (Fig 8C). Our findings support recent arguments that the UPR activates the CWI pathway in some pathogenic fungi. To the best of our knowledge, this is the first study to report that inhibition of the ER stress pathway exerts harmful effects on C. glabrata under cell wall stress.

Experimental Procedures

Strains and culture conditions

The C. glabrata strains used in this study are listed in S1 Table. A transactivator-expressing HETS202 strain was used to generate tet-KRE5 strains [70]. C. glabrata strains were
During normal cell growth, Kre5p serves as a chaperone cycle member for the proper folding of N-linked glycoprotein required for the cell wall synthesis in ER. (B) CgKRE5 repression induces CWI and UPR. The calcineurin pathway acts as a coordinating role for maintaining an appropriate chitin content in CgKRE5 repression-mediated CWI. (C) FK-506 prevents the calcineurin pathway and induces an imbalance in cell wall structure.

doi:10.1371/journal.pone.0161371.g008
grown at 37°C on a YPD complex medium containing 2% glucose, 2% peptone (Kyokuto, Japan), and 1% yeast extract (Nacalai Tesque, Japan). YPD agar plates were prepared by adding 2% agar (Nacalai Tesque) to the YPD medium. A yeast nitrogen base (0.67% YNB [Difco Laboratories, United States]) containing 2% glucose and 2% agar with appropriate amino acids and bases was used as a selective medium after HETS202 cells were transformed. Yeast transformations were performed using a modified lithium acetate method [71].

**Generation of conditional mutants**

Transformation of *C. glabrata* was performed as previously described [70]. Primers for strain construction were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus) and are listed in S2 Table. HETS202 cells were transformed using a DNA cassette prepared by PCR with primers p12874F and p12874TR for tet-*KRE5* and with plasmid pTK916-97t as a template. Insertion of the DNA cassette upstream of the *KRE5* ORFs in transformed cells was verified by colony PCR with primer p22F and p12874tcheck and Southern blot analysis. The Tet-*KRE5ΔSLT2* strain was constructed from the tet-*KRE5* strain. Approximately 1.0 kb of DNA from the 5´ and 3´UTRs flanking the *CgSLT2* coding sequence was amplified by PCR with the genomic DNA of *C. glabrata* as a template and with primers CgSLT2 5Fw and CgSLT2 5Rv + hphMX, and CgSLT2 3Rv + hphMX and CgSLT2 3Rv. A gene replacement cassette was constructed by performing fusion PCR with each PCR fragment and hphMX with primers CgSLT2 3rdFw and CgSLT2 3rdRv. Homologous recombination in the transformants was confirmed by PCR with primers CgSLT2 5Fw and CgSLT2 3Rv.

**Sequence analysis**

CAGL0E5412 and Sc*KRE5* were aligned using T-COFFEE version 11.00 (http://tcoffee.crg.cat) and were visualized using ESPript 3.0 (http://esprt.ibcp.fr/ESPript/ESPript/).

**Southern blotting analysis**

Southern blot analysis was performed following a standard protocol [72]. Genomic DNAs isolated from HETS202 and tet-*KRE5* strain were digested overnight with *Nde*I. Digested DNAs were separated in 0.8% agarose gel in 1× TAE buffer, and transferred to a Hybond-N+ membrane (GE Healthcare, United States). A PCR-amplified DNA fragment of *CgKRE5* was used as a probe (the primes used are listed in S2 Table). DNA probes were randomly labeled with [*α-32P]* dCTP (PerkinElmer, United States) by using the Random Primer DNA labeling kit ver.2 (Takara, Japan). Membranes were prehybridized for 1 h in 50 mL of 20× SSC solution and hybridized overnight at 65°C for the 32P-labeled probes. After hybridization, the membrane was rinsed twice with 2× SSC- 0.1% SDS solution at 65°C. The hybridized membrane was visualized in a BAS-5000 system (Fuji film, Japan). Raw data of the analysis are shown in S4 Fig.

**Drug susceptibility assay**

Drug susceptibility was determined using spot dilution test. Cells were cultured in liquid YPD medium at 37°C until they reached an exponential phase, and their density was adjusted to approximately 2 × 10^8 cells mL⁻¹. Next, 5 μL drops of serially diluted (1:5) cell suspension were spotted onto YPD agar plates containing the following: DOX (20 μg mL⁻¹; Sigma-Aldrich, United States), CFW (600 μg mL⁻¹; Sigma-Aldrich), CR (600 μg mL⁻¹; Sigma-Aldrich), TM (1.5 μg mL⁻¹; Nacalai Tesque), and FK-506 (100 ng mL⁻¹; Nacalai Tesque). The cells were cultured at 37°C for 48 h, and cell growth was observed.
Flow cytometry for cell cycle analysis

Flow cytometry for cell cycle analysis was conducted as described previously by Borah et al. [73], with few modifications. Logarithmically growing cells were harvested, washed twice with water, and fixed in 1 mL of ice-cold 70% ethanol at 4°C for 16 h. The fixed cells were washed with 1 mL of 50 mM sodium citrate buffer, suspended in 0.5 mL of 50 mM sodium citrate buffer containing 0.1 mg mL⁻¹ RNase A (Nippon Gene, Japan), and incubated at 37°C for 2 h. Propidium iodide (Nacalai Tesque) was added at a final concentration of 50 ng mL⁻¹. The cells were sonicated for 30 s to prevent doublet formation and were analyzed by flow cytometry with a FACSCalibur system (BD Biosciences, United States) at an excitation wavelength of 488 nm. A minimum of 20,000 events was recorded for each sample, and data were analyzed using CellQuest Pro software (BD Biosciences).

Preparation of alkali-insoluble fraction and analysis of cell wall composition

Cells were cultured in liquid YPD medium in the presence or absence of 20 μg mL⁻¹ DOX at 37°C until they reached the exponential phase. Next, the cells were washed 3 times with deionized water, collected by centrifugation, and extracted using 1% NaOH at 100°C for 24 h. The pellet obtained was washed twice with deionized water and extracted using 0.5 M acetic acid at 80°C for 24 h. The pellet was washed twice with deionized water and lyophilized.

The alkali-insoluble β-glucan content was determined as described by Umeyama et al. with some modifications. Briefly, 1.0 mg of lyophilized alkali-insoluble fraction was suspended in 10 mM Tris-HCl (pH 7.4) containing 1.0 mg mL⁻¹ Zymolyase-100T (Nacalai Tesque), and incubated at 37°C for 24 h. The precipitate obtained was removed by centrifugation at 15,500 × g for 10 min, and half of the supernatant was dialyzed overnight against 10 mM Tris-HCl (pH 7.4). The hexose content was determined using the phenol H₂SO₄ method.

The total cell wall chitin content was determined as described by François with some modifications [74]. Briefly, 20 mg of lyophilized alkali-insoluble fraction was hydrolyzed using 1 M H₂SO₄ at 100°C for 4 h and neutralized. The precipitate obtained was removed by centrifugation, and the supernatant obtained was dissolved in 2 mL of deionized water after evaporation to dryness. Next, 500 μL of the solution was added to 1 mL of acetylacetone solution (10% [v/v] acetylacetone in 1.25 M sodium carbonate) and incubated at 90°C for 1 h, followed by addition of 10 mL of 100% ethanol and 1 mL of Reissig reagent (1% 4-dimethylaminobenzaldehyde, 1.25% [v/v] HCl in glacial acetic acid). The chitin content was determined by measuring absorbance of glucosamine at 490 nm.

mRNA extraction and reverse transcription-PCR

Logarithmic-phase cells (OD₆₀₀ = 0.1) were inoculated in YPD medium supplemented with or without the required chemicals and were grown at 37°C for 4 h. For reverse transcription-PCR, total RNA was isolated using Sepazol RNA I Super G (Nacalai Tesque) according to the manufacturer’s instructions. The isolated RNA was treated with DNase I to remove any residual DNA, and 500 ng of total RNA was reverse transcribed to cDNA by using ReverTra Ace qPCR Master mix (Toyobo, Japan). Next, 1 μL of the resulting RT reaction mixture was used as a template for performing individual PCR with Thunderbird SYBR qPCR mix (Toyobo). Real-time PCR was performed in triplicate in a 96-well plate by using a StepOnePlus Real-Time PCR System (Applied Biosystems, United States). Relative expression ratios were calculated using the ΔΔCt method. PGK1 was used as a normalization reference for determining target gene expression level, and DMSO-treated HETS 202 cells were used as calibrators in each
experiment. Primers for real-time PCR were designed using Primer3Plus and are listed in S2 Table. The assays were repeated at least twice independently.

**Western blotting analysis**

Logarithmic-phase cells were inoculated in YPD medium and incubated at 37°C for 4 h. Next, the cells were washed twice with ice-cold deionized H2O and suspended in 200 μL of homogenizing buffer (50 mM Tris [pH 7.5] and 1 mM EDTA) containing 1× PhosSTOP (Roche, Switzerland) and 1× Complete (EDTA-free; Roche). The cells were then lysed by using glass beads and vortexing at the maximum speed. Cell debris and unbroken cells were removed by centrifugation at 13,000 ×g and 4°C for 10 min. Protein content was quantified using a XL-Bradford protein assay kit (APRO Science, Japan) per the supplier’s instructions. Next, 40 μg of the total protein was resolved by SDS-PAGE on a 10% gel and transferred onto PVDF membranes (PALL Life Science) that were then blocked using Blocking One-P (Nacalai Tesque) for 1 h at room temperature. Immunoblotting was conducted using an anti-phosphorylated-p44/42 MAP kinase antibody (#4370; Cell Signaling Technology, United States) and anti-Pgk1p antibody (Abcam, United Kingdom) at a dilution of 1:10,000 in TBS and 0.1% Tween 20 for 1 h at room temperature. HRP-linked anti-rabbit IgG (Promega, United States) was used as the secondary antibody and the blots were developed using an ECL plus western blotting detection system (GE Healthcare). The assays were repeated at least twice independently. The raw data of each analysis are shown in S5 Fig.

**Microscopy**

Logarithmic-phase cells (OD600 of 0.1) were inoculated in YPD medium supplemented with or without the required chemicals and grown at 37°C for 4 h. The cells were harvested, washed twice with water, and fixed using 1 mL of 4% paraformaldehyde in phosphate-buffered saline at 4°C for 16 h. The cells were then embedded in Permafluor (Thermo scientific, United States) on a slide glass and observed under a differential interference contrast microscope (BX-53; Olympus; Japan). All images were acquired under identical conditions and processed in parallel.

**Supporting Information**

S1 Fig. Sequence analysis of CgKRE5. (EPS)

S2 Fig. Complementation of CgKRE5 knockdown/knockout by expressing ScKre5p. (A) Homologous recombination with CgKRE5 gene disruption cassette was performed under ScKre5p expressing mutant. A ScKre5p expression vector pGRB2.2-ScKRE5 or an empty vector pGRB2.2 was transformed in C. glabrata strain NAU3. CgKRE5 gene disruption cassette was then transformed in these strains and observed whether transformants were obtained. (B) Spot dilution assay was performed using ScKre5p expression tet-KRE5 mutant, tet-KRE5-URA3 pGRB2.2-ScKRE5. Empty vector pGRB2.2 was also transformed in tet-KRE5-URA3 (tet-KRE5ΔURA3 pGRB2.2). Five-microliter suspensions at an OD of 0.1 and serially diluted (1: 5) cells were spotted on SC URA- plates with the indicated concentration of reagents incubated at 37°C. (PDF)

S3 Fig. Knock out Confirmation of SLT2 gene in tet-KRE5 strain. (EPS)
S4 Fig. Original blots data for Southern blot analysis.
(EPS)

S5 Fig. Original blots data for western blot analysis.
(EPS)

S1 Table. Strain list used in this study.
(XLSX)

S2 Table. Primer list used in this study.
(XLSX)

Acknowledgments
We thank Dr. H. Nakayama and N. Ishizaki (Faculty of Pharmaceutical Sciences, Suzuka University of Medical Sciences, Mie, Japan) for providing \textit{C. glabrata} strain NAU3 \cite{75} and Dr. Brendan Cormack for providing pGRB2.2 (Addgene plasmid #45342).

Author Contributions
Conceptualization: YT NS.
Data curation: MS FI HC NS.
Formal analysis: YT TA.
Investigation: YT TA MS-O.
Methodology: YT MS FI NS.
Project administration: YT MS NS.
Resources: YT MS-O AT-N HC.
Supervision: MS HC NS.
Validation: YT.
Writing - original draft: YT.
Writing - review & editing: MS HC NS.

References
1. Birkaya B, Maddi A, Joshi J, Free SJ, Cullen PJ. Role of the cell wall integrity and filamentous growth mitogen-activated protein kinase pathways in cell wall remodeling during filamentous growth. \textit{Eukaryot Cell}. 2009; 8: 1118–1133. doi:10.1128/EC.00006-09 PMID: 19502582
2. Levin DE. Cell Wall Integrity Signaling in \textit{Saccharomyces cerevisiae}. \textit{Cell Wall Integrity Signaling in \textit{Saccharomyces cerevisiae}}. \textit{Microbiol Mol Biol Rev}. 2005; 69: 262–291. doi:10.1128/MMBR.69.2.262 PMID: 15944456
3. Scrimale T, Didone L, de Mesy Bentley KL, Krysan DJ. The unfolded protein response is induced by the cell wall integrity mitogen-activated protein kinase signaling cascade and is required for cell wall integrity in \textit{Saccharomyces cerevisiae}. \textit{Mol Biol Cell}. 2009; 20: 164–75. doi:10.1091/mbc.E08-08-0809 PMID: 19871375
4. Nett JE, Sanchez H, Cain MT, Ross KM, Andes DR. Interface of \textit{Candida albicans} biofilm matrix-associated drug resistance and cell wall integrity regulation. \textit{Eukaryot Cell}. 2011; 10: 1660–1669. doi:10.1128/EC.05126-11 PMID: 21666076
5. Valiante V, Jain R, Heinekamp T, Brakhage AA. The MpkA MAP kinase module regulates cell wall integrity signaling and pyomelanin formation in \textit{Aspergillus fumigatus}. \textit{Fungal Genet Biol}. 2009; 46: 909–918. doi:10.1016/j.fgb.2009.08.005 PMID: 19715768
6. Jung US, Levin DE. Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. Mol Microbiol. 1999; 34: 1049–1057. PMID: 10594829

7. Cid VJ, Cenamor R, Sánchez M, Nombela C. A mutation in the Rho1-GAP-encoding gene BEM2 of Saccharomyces cerevisiae affects morphogenesis and cell wall functionality. Microbiology. 1998; 144: 25–36. doi: 10.1099/00221287-144-1-25 PMID: 9467898

8. Levinson JN, Shahinian S, Sdicu AM, Tessier DC, Bussey H. Functional, comparative and cell biological analysis of Saccharomyces cerevisiae Kre5p. Yeast. 2002; 19: 1243–1259. doi: 10.1002/yea.908 PMID: 12271460

9. Chen RE, Chen RE, Thorner J, Thorner J. Function and regulation in MAPK signaling pathways: lessons learned from the yeast Saccharomyces cerevisiae. Biochim Biophys Acta. 2007; 1773: 1311–40. doi: 10.1016/j.bbamcr.2007.05.003 PMID: 17604854

10. Miyazaki T, Inamine T, Yamauchi S, Nagayoshi Y, Sajo T, Izumikawa K, et al. Role of the Slt2 mitogen-activated protein kinase pathway in cell wall integrity and virulence in Candida glabrata. FEMS Yeast Res. 2010; 10: 343–352. doi: 10.1111/j.1567-1364.2010.00611.x PMID: 20214686

11. Jandric Z, Gregori C, Klopf E, Radolf M, Schüller C. Sorbic acid stress activates the Candida glabrata high osmolarity glycerol MAP kinase pathway. Front Microbiol. 2013; 4. doi: 10.3389/fmicb.2013.00350

12. Barchiesi F, Spreghini E, Tomassetti S, Giannini D, Scalise G. Caspofungin in combination with amphotericin B against Candida parapsilosis. Antimicrob Agents Chemother. 2007; 51: 941–945. doi: 10.1128/AAC.00880-06 PMID: 17158939

13. Clancy CJ, Huang H, Cheng S, Derendorf H, Nguyen MH. Characterizing the effects of caspofungin on Candida albicans, Candida parapsilosis, and Candida glabrata isolates by simultaneous time-kill and postantifungal-effect experiments. Antimicrob Agents Chemother. 2006; 50: 2569–2572. doi: 10.1128/AAC.00291-06 PMID: 16801448

14. Kolar R, Reinhold BB, Petakova E, Ashwell G, Kapteyn JC, et al. Architecture of the Yeast Cell Wall. J Biol Chem. 1997; 272: 17762–17775. PMID: 9211929

15. Shahinian S, Bussey H. beta-1,6-Glucan synthesis in Saccharomyces cerevisiae. Mol Microbiol. 2000; 35: 477–489. 1713 [pii] PMID: 10672173

16. De Groot PWJ, Kraneveld EA, Qing YY, Dekker HL, Groß U, Crielaard W, et al. The cell wall of the human pathogen Candida glabrata: Differential incorporation of novel adhesin-like wall proteins. Eukaryot Cell. 2008; 7: 1951–1964. doi: 10.1128/EC.00284-08 PMID: 18806209

17. Roetzer A, Gabaldón T, Schüller C. From Saccharomyces cerevisiae to Candida glabrata in a few easy steps: Important adaptations for an opportunistic pathogen. FEMS Microbiology Letters. 2011. pp. 1–9. doi: 10.1111/j.1574-6968.2010.02102.x

18. Kollár R, Reinhold BB, Petakova E, Ashwell G, Kapteyn JC, et al. Architecture of the Yeast Cell Wall. J Biol Chem. 1997; 272: 17762–17775. PMID: 9211929

19. Ahmad KM, Koko??ar J, Guo X, Gu Z, Ishchuk OP, Pi??kur J. Genome structure and dynamics of the yeast pathogen Candida glabrata. FEMS Yeast Res. 2014; 14: 529–535. doi: 10.1111/1567-1364.12145 PMID: 24528571

20. Feng X, Krishnan K, Richie DL, Almanianda V, Hartl L, Grahm N, et al. Haca-independent functions of the ER stress sensor irea synergize with the canonical UPR to influence virulence traits in Aspergillus fumigatus. PLoS Pathog. 2011; 7: doi: 10.1371/journal.ppat.1002330

21. Jung KW, Kang HA, Bahn YS. Essential Roles of the Kar2/BiP Molecular Chaperone Downstream of the UPR Pathway in Cryptococcus neoformans. PLoS One. 2013; 8. doi: 10.1371/journal.pone.0058956

22. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. Science. 2011; 334: 1081–6. doi: 10.1126/science.1209038 PMID: 22116877

23. Gardiner BM, Walter P. Unfolded Proteins are Ire1-Activating Ligands that Directly Induce the Unfolded Protein Response. Science (80-). 2011; 333: 1891–1895. doi: 10.1126/science.1209126
27. Nikawa J, Yamashita S. IRE1 encodes a putative protein kinase containing a membrane-spanning domain and is required for inositol phototrophy in Saccharomyces cerevisiae. Mol Microbiol. 1992; 6: 1441–1446. doi: 10.1111/j.1365-2958.1992.tb00864.x PMID: 1625574

28. Back SH, Schr?der M, Lee K, Zhang K, Kaufman RJ. ER stress signaling by regulated splicing: IRE1/ NIKawa J, Yamashita S. IRE1 encodes a putative protein kinase containing a membrane-spanning domain and is required for inositol phototrophy in Saccharomyces cerevisiae. Mol Microbiol. 1992; 6: 1441–1446. doi: 10.1111/j.1365-2958.1992.tb00864.x PMID: 1625574

29. Urano F, Bertolotti a, Ron D. IRE1 and efferent signaling from the endoplasmic reticulum. J Cell Sci. 2000; 113 Pt 21: 3697–3702. PMID: 11034898

30. Miyazaki T, Kohno S. ER stress response mechanisms in the pathogenic yeast Candida glabrata and their roles in virulence. Virulence. 2014; 5: 365–70. doi:10.4161/viru.27373 PMID: 24335436

31. Miyazaki T, Nakayama H, Nagayoshi Y, Kakeya H, Kohno S. Dissection of Ire1 functions reveals stress response mechanisms uniquely evolved in Candida glabrata. PLoS Pathog. 2013; 9: e1003160. doi: 10.1371/journal.ppat.1003160 PMID: 23382685

32. Meaden P, Hill K, Wagner J, Slípetz D, Sommer SS, Bussey H. The yeast KRE5 gene encodes a probable endoplasmic reticulum protein required for (1–6)-beta-D-glucan synthesis and normal cell growth. Mol Cell Biol. 1990; 10: 3013–3019. PMID: 2188106

33. Herrero AB, Magnelli P, Mansour MK, Levitz SM, Bussey H, Aebi M, et al. Involvement of protein N-glycosidase chain glucosylation and processing in the biosynthesis of cell wall beta -1,6-Glucan of Saccharomyces cerevisiae. Genetics. 1998; 149: 843–856. PMID: 9611196

34. Roetzler A, Gabaldón T, Schüller C. From Saccharomyces cerevisiae to Candida glabrata in a few easy steps: important adaptations for an opportunistic pathogen. FEMS Microbiol Lett. 2010; doi:10.1111/j.1365-2958.1992.tb00864.x PMID: 1625574

35. Fanchiotti S, Fernández F, D’Alessio C, Parodi AJ. The UDP-Glc:glycoprotein glucosyltransferase homologues have distinct biological functions. PLoS One. 2011; 6. doi: 10.1371/journal.pone.0027025

36. Buzzi LI, Simonetta SH, Parodi AJ, Castro OA. The two Caenorhabditis elegans UDP-glucose:Glycoprotein glucosyltransferase is required for inositol phototrophy in Saccharomyces cerevisiae. Mol Microbiol. 1992; 6: 2000; 113 Pt 21: 3697–3702. PMID: 11034898

37. Roetzler A, Gabaldón T, Schüller C. From Saccharomyces cerevisiae to Candida glabrata in a few easy steps: important adaptations for an opportunistic pathogen. FEMS Microbiol Lett. 2010; doi:10.1111/j.1365-2958.1992.tb00864.x PMID: 1625574

38. Selvakumar D, Miyamoto M, Furuichi Y, Komiyama T. Inhibition of fungal??-1,3-Glucan synthase and normal cell growth by HM-1 killer toxin single-chain anti-idiotypic antibodies. Antimicrob Agents Chemother. 1996; 30: 3090–3097. doi:10.1128/AAC.30.11.3090-3097.20.2005 PMID: 15944456

39. Roetzler A, Gabaldón T, Schüller C. From Saccharomyces cerevisiae to Candida glabrata in a few easy steps: important adaptations for an opportunistic pathogen. FEMS Microbiol Lett. 2010; doi:10.1111/j.1365-2958.1992.tb00864.x PMID: 1625574

40. Roncero C, Duran A. Effect of Calcofluor White and Congo red on fungal cell wall morphogenesis: In vivo activation of chitin polymerization. J Bacteriol. 1985; 163: 1180–1185. PMID: 3897187

41. Bermejo C, Rodríguez E, García R, Rodríguez-Peña JM, Rodríguez de la Concepción ML, Rivas C, et al. The sequential activation of the yeast HOG and SLT2 pathways is required for cell survival to cell wall stress. Mol Biol Cell. 2008; 19: 1113–1124. doi:10.1091/mbc.E07-08-0742 PMID: 18184748

42. Elorza M V, Rico H, Sentandreu R. Calcofluor white alters the assembly of chitin fibrils in Saccharomyces cerevisiae and Candida albicans cells. J Gen Microbiol. 1983; 129: 1577–1582. doi: 10.1099/00221287-129-5-1577 PMID: 6352868

43. Levin DE. Cell wall integrity signaling in Saccharomyces cerevisiae. Microbiol Mol Biol Rev. 2005; 69: 262–291. doi:10.1128/MMBR.69.2.262–291.2005 PMID: 15944456

44. Miyazaki T, Nakayama H, Nagayoshi Y, Kakeya H, Kohno S. Dissection of Ire1 Functions Reveals Stress Response Mechanisms Uniquely Evolved in Candida glabrata. PLoS Pathog. 2013; 9. doi: 10.1371/journal.ppat.1003160
48. Munro CA, Winter K, Buchan A, Henry K, Becker JM, Brown AJP, et al. Chs1 of Candida albicans is an essential chitin synthase required for synthesis of the septum and for cell integrity. Mol Microbiol. 2001; 39: 1414–1426. doi: 10.1046/j.1365-2958.2001.02347.x PMID: 11251855

49. Lagorce A, Berre-Anton V Le, Aguilar-Uscanga B, Martin-Yken H, Dagkessamanskaia A, François J. Involvement of GFA1, which encodes glutamine-fructose-6-phosphate amidotransferase, in the activation of the chitin synthesis pathway in response to cell-wall defects in Saccharomyces cerevisiae. Eur J Biochem. 2002; 269: 1697–1707. doi: 10.1046/j.1432-1327.2002.02814.x PMID: 11895440

50. Chavan M, Suzuki T, Rekowicz M, Lennarz W. Genetic, biochemical, and morphological evidence for the involvement of N-glycosylation in biosynthesis of the cell wall beta1,6-glucan of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A. 2003; 100: 15381–15386. doi: 10.1073/pnas.2536661100 PMID: 14676317

51. Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature. 1999; 397: 271–274. doi: 10.1038/16729 PMID: 9930704

52. Kurita T, Noda Y, Yoda K. Action of multiple endoplasmic reticulum chaperon-like proteins is required for proper folding and polarized localization of Kre6 protein essential in yeast cell wall beta1,6-glucan synthesis. J Biol Chem. 2012; 287: 17415–17424. doi: 10.1074/jbc.M111.321016 PMID: 22447934

53. Scrimale T, Didone L, de Mesy Bentley KL, Krysan DJ. The unfolded protein response is induced by the cell wall integrity mitogen-activated protein kinase signaling cascade and is required for cell wall integrity in Saccharomyces cerevisiae. Mol Biol Cell. 2009; 20: 164–175. doi: 10.1091/mbc.E08 PMID: 18971375

54. Frost A, Elgort MG, Brandman O, Ives C, Collins SR, Miller-Vedam L, et al. Functional repurposing revealed by comparing S. pombe and S. cerevisiae genetic interactions. Cell. 2012; 149: 1339–1352. doi: 10.1016/j.cell.2012.04.028 PMID: 22682253

55. Chamilos G, Lewis RE, Albert N, Kontoyiannis DP. Paradoxical effect of echinocandins across Candida species in vitro: Evidence for Echinocandin-Specific and Candida species-related differences. Antimicrob Agents Chemother. 2007; 51: 2257–2259. doi: 10.1128/AAC.00097-07 PMID: 17438060

56. Stevens DA, Ichinomiya M, Koshi Y, Horuchi H. Escape of Candida from caspofungin inhibition at concentrations above the MIC (paradoxical effect) accomplished by increased cell wall chitin; evidence for beta-1,6-glucan synthesis inhibition by caspofungin. Antimicrob Agents Chemother. 2008; 50: 3160–3161. doi: 10.1128/AAC.00563-06 PMID: 18940118

57. Cota JM, Grabinski JL, Talbert RL, Burgess DS, Rogers PD, Edlind TD, et al. Increases in SLT2 expression and chitin content are associated with incomplete killing of Candida glabrata by caspofungin. Antimicrob Agents Chemother. 2009; 52: 1144–1146. doi: 10.1128/AAC.01542-07 PMID: 18086838

58. Katiyar S, Pfaller M, Edlind T. Candida albicans and Candida glabrata clinical isolates exhibiting reduced echinocandin susceptibility. Antimicrob Agents Chemother. 2006; 50: 2892–2894. doi: 10.1128/AAC.00349-06 PMID: 16870797

59. Kollár R, Reinhold BB, Petráková E, Yeh HJ, Ashwell G, Drgonová J, et al. Architecture of the yeast cell wall. Beta(1,3)-glucan, and chitin. J Biol Chem. 1997; 272: 17762–17775. doi: 10.1074/jbc.272.28.17762 PMID: 9211929

60. Hurtado-Guerrero R, Schüttelkopf AW, Mouyina I, Ibrahim AFM, Shepherd S, Fontaine T, et al. Molecular mechanisms of yeast cell wall glucan remodeling. J Biol Chem. 2009; 284: 8461–8469. doi: 10.1074/jbc.M807990200 PMID: 19097997

61. Springer DJ, Chen Y-L, Abraham SN, Regev A, Thompson DA, Heitman J, et al. Convergent Evolution of Calcineurin Pathway Roles in Thermotolerance and Virulence in Candida glabrata. G3;#58; Genes|Genomes|Genetics. 2012. pp. 675–691. doi: 10.1534/g3.112.002279

62. Miyazaki T, Izuimakawa K, Nagayoshi Y, Saijo T, Yamauchi S, Morinaga Y, et al. Functional characterization of the regulators of calcineurin in Candida glabrata. FEMS Yeast Res. 2011; 11: 621–30. doi: 10.1111/j.1567-1364.2011.00751.x PMID: 22093746

63. Denardi LB, Mario DAN, Loreto ÉS, Santurio JM, Alves SH. Synergistic effects of tacrolimus and azole antifungal compounds in fluconazole-susceptible and fluconazole-resistant Candida glabrata isolates. Braz J Microbiol. 2015; 46: 125–9. doi: 10.1590/S1518-83872015000100025 PMID: 26221097

64. Skeens M, Pai V, Garee A, Termuhlen AM, Bajwa RPS, Gross TG, et al. Twice daily i.v. bolus tacrolimus infusion for GVHD prophylaxis in children undergoing stem cell transplantation. Bone Marrow Transplantation. 2012. doi: 10.1038/bmt.2012.59

65. Ram R, Gaffer-Gvili A, Yeshurun M, Paul M, Raanani P, Shpilberg O. Prophylaxis regimens for GVHD: systematic review and meta-analysis. Bone Marrow Transplant. 2009; 43: 643–653. doi: 10.1038/bmt.2008.373 PMID: 18997826
66. Neofytos D, Fishman JA, Horn D, Anaissie E, Chang C-H, Olyaei A, et al. Epidemiology and outcome of invasive fungal infections in solid organ transplant recipients. Transpl Infect Dis. 2010; 12: 220–229. doi: 10.1111/j.1399-3062.2010.00492.x PMID: 20113459

67. Proia L, Miller R. Endemic fungal infections in solid organ transplant recipients. Am J Transplant. 2009; 9 Suppl 4: S199–S207. doi: 10.1111/j.1600-6143.2009.02912.x PMID: 20070682

68. Sugita T, Tajima M, Ito T, Saito M, Tsuboi R, Nishikawa A. Antifungal activities of tacrolimus and azole agents against the eleven currently accepted Malassezia species. J Clin Microbiol. 2005; 43: 2824–2829. doi: 10.1128/JCM.43.6.2824–2829.2005 PMID: 15956404

69. Maesaki S, Marichal P, Hossain MA, Sanglard D, Bossche H Vanden, Kohno S. Synergic effects of tacrolimus and azole antifungal agents against azole-resistant Candida albicans strains. J Antimicrob Chemother. 1998; 42: 747–753. doi: 10.1093/jac/42.6.747 PMID: 10052898

70. Ueno K, Uno J, Nakayama H, Sasamoto K, Mikami Y, Chibana H. Development of a highly efficient gene targeting system induced by transient repression of YKU80 expression in Candida glabrata. Eukaryot Cell. 2007; 6: 1239–1247. doi: 10.1128/EC.00414-06 PMID: 17513567

71. Agatep R, Kirkpatrick RD, Parchaliuk DL, Woods RA, Gietz RD. Transformation of Saccharomyces cerevisiae by the lithium acetate/single-stranded carrier DNA/polyethylene glycol protocol. Technical Tips Online. 1998. pp. 133–137. doi:10.1016/S1366-2120(08)70121-1

72. Gebbie L. Genomic Southern blot analysis. Methods Mol Biol. 2014; 1099: 159–77. doi: 10.1007/978-1-62703-715-0_14 PMID: 24243203

73. Borah S, Shivarathri R, Kaur R. The Rho1 GTPase-activating protein CgBem2 is required for survival of azole stress in Candida glabrata. J Biol Chem. 2011; 286: 34311–34324. doi: 10.1074/jbc.M111.264671 PMID: 21832071

74. François JM. A simple method for quantitative determination of polysaccharides in fungal cell walls. Nat Protoc. 2006; 1: 2995–3000. doi: 10.1038/nprot.2006.457 PMID: 17406560

75. Niimi K, Woods MA, Maki K, Nakayama H, Hatakenaka K, Chibana H, et al. Reconstitution of high-level micafungin resistance detected in a clinical isolate of Candida glabrata identifies functional homozygosity in glucan synthase gene expression. J Antimicrob Chemother. 2012; 67: 1666–1676. doi: 10.1093/jac/dks112 PMID: 22514266