Overexpression of *Candida albicans* Secreted Aspartyl Proteinase 2 or 5 Is Not Sufficient for Exacerbation of Immunopathology in a Murine Model of Vaginitis

Hubertine M. E. Willems, Winter S. Bruner, Katherine S. Barker, Junyan Liu, Glen E. Palmer, Brian M. Peters

Department of Clinical Pharmacy and Translational Science, College of Pharmacy, University of Tennessee Health Science Center, Memphis, Tennessee, USA

ABSTRACT The secreted aspartyl proteinases of *Candida albicans* have long been implicated in virulence at the mucosal surface, including contributions to colonization and immunopathogenesis during vulvovaginal candidiasis. In an effort to disentangle hypha-associated virulence factor regulation from morphological transition, the purpose of this study was to determine if overexpression of *SAP2* or *SAP5* in an *efg1Δ/Δ cph1Δ/Δ* mutant could restore the capacity to cause immunopathology during murine vaginitis to this avirulent hypofilamentous strain. Two similar yet distinct genetic approaches were used to construct expression vectors to achieve *SAP* overexpression, and both genetic and functional assays confirmed elevated *SAP* activity in transformed strains. Similar to previous findings, intravaginal challenge of C57BL/6 mice with hypha-defective strains attained high levels of mucosal colonization but failed to induce robust vaginal immunopathology (neutrophil recruitment, interleukin-1 β [IL-1 β] secretion, and lactate dehydrogenase release) compared to that with the hypha-competent control. Moreover, constitutive expression of *SAP2* or *SAP5* in two distinct sets of such strains did not elicit immunopathological markers at levels above those observed during challenge with isogenic empty vector controls. Therefore, these results suggest that the physiological contributions of *SAPs* to vaginal immunopathology require hypha formation, other hypha-associated factors, or genetic interaction with *EFG1* and/or *CPH1* to cause symptomatic infection. Additionally, the outlined expression strategy and strain sets will be useful for decoupling other downstream morphogenetic factors from hyphal growth.

KEYWORDS *Candida*, *SAPs*, fungal, pathogenesis, vaginitis, virulence factors, vulvovaginal

Vulvovaginal candidiasis (VVC), caused by the polymorphic fungal pathogen *Candida albicans*, is the most prevalent human fungal infection, affecting approximately 75% of women at least once in their lifetime, primarily in their childbearing years (1). Moreover, it is estimated that roughly 5 to 8% of all women suffer from recurrent infection, defined as 3 or more symptomatic episodes per year, requiring continuous antifungal maintenance therapy to prevent infectious relapse (2). Despite its high incidence rate and the significant insight into host response mechanisms that contribute to disease pathogenesis, still relatively little is understood about the fungal virulence factors that govern symptomatic immunopathology.

The clinically relevant mouse model of vaginal candidiasis, along with robust approaches to genetically manipulate *C. albicans*, has been an indispensable tool for

Received 6 April 2017 Returned for modification 21 April 2017 Accepted 26 July 2017

Accepted manuscript posted online 31 July 2017

Citation Willems HME, Bruner WS, Barker KS, Liu J, Palmer GE, Peters BM. 2017.

Overexpression of *Candida albicans* secreted aspartyl proteinase 2 or 5 is not sufficient for exacerbation of immunopathology in a murine model of vaginitis. Infect Immun 85:e00248-17. <https://doi.org/10.1128/IAI.00248-17>.

Editor George S. Deepe, University of Cincinnati

Copyright © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Brian M. Peters, brian.peters@uthsc.edu.

mechanistically exploring this exceedingly common host-pathogen interaction. A live-challenge study conducted on volunteer women by Fidel et al. determined that symptomatic infection was linked with the presence of neutrophils in the vaginal lavage fluid, as women colonized to similar levels (but without neutrophil recruitment) lacked disease symptoms (3). This finding ultimately led to a paradigm shift in the philosophy of vaginitis pathogenesis, characterizing VVC as an immunopathology in which the host response drives symptomatic disease. Indeed, our lab previously demonstrated that the ability of *C. albicans* to switch from ovoid yeast cells to elongated hyphae is crucial for the induction of immunopathology, including neutrophil recruitment, innate inflammatory cytokine production (e.g., S100A8 and interleukin-1 β [IL-1 β]), and mucosal damage (lactate dehydrogenase [LDH] release) (4). Strains genetically engineered to largely inhibit hypha formation (herein defined by the term “hypha defective”) colonized the murine vagina at high levels yet failed to induce robust immunopathology, essentially mimicking asymptomatic colonization and somewhat recapitulating findings from the aforementioned live-challenge study.

The yeast-to-hypha switch not only induces an obvious morphological change but also is associated with a complete transcriptomic reprogramming of the fungal cell, including the expression of many hypha-specific virulence factors (5). In an effort to better understand host and fungal gene expression at the murine vaginal mucosa, we previously conducted an *in vivo* transcriptome sequencing (RNA-seq) study (6). Using analysis of the number of reads per kilobase per million (RPKM), approximately 50 of the most highly expressed fungal genes were elucidated. Among these were the known hypha-associated factors *ECE1*, *HWP1*, *HYR1*, and *SAP5*. Due to its previously identified consistently high expression in tissue culture and clinical vaginal samples and its role in mucosal pathogenesis, the secreted aspartyl proteinase Sap5 was further investigated for its role in VVC (7, 8). Indeed, deletion of *SAP5* alone (or deletion of a triad of hypha-associated SAP genes [*SAP4*, -5, and -6]) resulted in a modest but significant reduction in immunopathology *in vivo*, purportedly by reducing NLRP3 inflammasome activation (6).

In *C. albicans*, the secreted aspartyl proteinase (SAP) family is encoded by 10 individual SAP genes, named numerically, with Sap1 to -8 secreted into the extracellular environment and Sap9 and -10 being cell wall associated (9). Although the morphological association of *SAP7* to -10 is still somewhat unclear, expression of *SAP1* to -3 is primarily linked with yeast forms of *C. albicans*, while expression of *SAP4* to -6 is predominantly associated with hyphal forms (10). SAPs serve two prominent roles: their believed main biological function is enzymatic digestion of complex extracellular proteins that can then be utilized as sources of nutrition, and their secondary (perhaps unintended) function is involved in virulence, including innate immune signaling, damage, and enhanced colonization (9). Indeed, using *in vivo* expression technology (IVET), *SAP4* and *SAP5* were determined to be expressed in the murine vagina at time points consistent with immunopathology onset following *C. albicans* challenge (11). Moreover, enzymatic inhibition of, genetic disruption of, or immunization with Sap2 protein has long been suggested to result in reduced colonization of the rat vagina (12–15). Additionally, recent accumulating evidence suggests that SAPs may possess novel inflammatory roles independent of their enzymatic activity (16).

Therefore, the objective of this study was to determine whether overexpression of *SAP2* or *SAP5* in hypha-defective strains of *C. albicans* could induce immunopathology in an estrogen-dependent murine model of vaginitis. Furthermore, by reconstitution of hypha-associated factors in hypha-defective cells, we began to tease apart the necessary and sufficient roles of morphogenetic regulation and physical morphological transition in the immunopathogenesis of *C. albicans* at the vaginal mucosa.

RESULTS AND DISCUSSION

Previous work clearly demonstrated that deletion of *C. albicans EFG1* (alone or in tandem with *CPH1*) results in a blastoconidial growth phenotype *in vivo* and a significant impairment of immunopathogenesis at the mucosal surface (4, 17, 18). However,

because deletion of these transcription factors affects morphological transition as well as downstream associated fungal effectors, it is difficult (if not impossible) to distinguish which element plays a key role(s) in vaginitis pathogenesis. Therefore, we generated hypha-defective strains (i.e., those capable of colonizing without eliciting inflammation) that overexpress the hypha-associated gene *SAP5* and its yeast counterpart *SAP2* (previously identified as important in vaginitis pathogenesis) to determine whether these factors were sufficient to restore vaginopathogenic potential to the otherwise avirulent blastoconidial-dominant strain GF54F, a derivative of the widely characterized *efg1Δ/Δ cph1Δ/Δ* mutant HLC54 (19).

After transformation of GF54F with empty vector or plasmid pWB3 (see Fig. S1 in the supplemental material) containing the *SAP2* or *SAP5* open reading frame (ORF) driven by the constitutive *TEF1* promoter, we performed microscopy experiments to determine the morphology after growth in RPMI medium. The results confirmed that all GF54F variants remained in the yeast form, in contrast to wild-type (WT) strain GP1, which formed hyphae as expected (Fig. 1A). We also wished to determine if SAPs were indeed being overexpressed at the gene level. Therefore, strains containing empty vector (GF54F+pWB3) or SAP overexpression constructs (GF54F+SAP2 and GF54F+SAP5) were grown in yeast extract-peptone-dextrose (YPD) to suppress residual endogenous SAP activity, and quantitative PCR (qPCR) was performed to assess both *SAP2* and *SAP5* expression. The findings indicated that *SAP2* and *SAP5* were overexpressed approximately 100-fold and 200-fold, respectively, in each target strain *in vitro* relative to the levels in the empty vector control (Fig. 1B). This difference in expression could be due to slightly elevated levels of *SAP2* (compared to those of hypha-specific *SAP5*) in the control strain. Strains denoted "TF1" or "TF2" simply indicate multiple clones obtained by independent transformations (Fig. 1B). Importantly, expression patterns were target gene specific, as *SAP2* (open bars) or *SAP5* (hatched bars) was upregulated only in strains containing the respective overexpression construct. Lastly, enhanced SAP expression at the protein level was verified by a functional bovine serum albumin (BSA) degradation assay in which secreted SAPs create a hazy zone of clearance around the fungal colony when incubated on yeast extract (YE)-BSA agar (20). Strain GF54F+pWB3 demonstrated little to no SAP activity, while discernible zones of clearance were observed for both the GF54F+SAP2 and GF54F+SAP5 overexpression constructs (Fig. 1C). These results are consistent with previous studies in which the *efg1Δ/Δ cph1Δ/Δ* mutant HLC54 expressed no *SAP1* to -8 and greatly reduced *SAP9* and -10 when inoculated onto reconstituted oral epithelia, concomitant with a lack of damage and innate immune signaling (21).

Moreover, these phenotypes were confirmed *in vivo* at the vaginal mucosa, as *C. albicans* strains harboring vector control (Fig. 2B), *SAP2* overexpression (Fig. 2C), and *SAP5* overexpression (Fig. 2D) constructs grew largely as blastoconidial forms, in contrast to GP1, which avidly formed hyphae (Fig. 2A). Expression analyses of RNAs isolated from vaginal lavage cells (including colonizing *C. albicans*) revealed selective SAP expression patterns similar to those observed *in vitro*, achieving 30- and 15-fold increases for *SAP2* and *SAP5*, respectively, relative to the vector control level (Fig. 2E). Rational selection of the *TEF1* promoter to drive gene expression *in vivo* was based on our previously published data demonstrating high expression of this gene at the vaginal mucosa (6). Importantly, we also compared SAP expression levels in our hypha-defective strains to those in strain GP1 *in vivo* and determined that *SAP2* and *SAP5* were indeed overexpressed 8-fold and 3-fold, respectively, relative to those in the WT control (Fig. 2F). A notable reduction in the magnitude of SAP overexpression from values obtained *in vitro* may be explained by elevated basal transcription in the vector control strain *in vivo*, mediated independently of the *EFG1* or *CPH1* transcription factor, or by inefficiency of gene-specific transcription in the presence of host RNA. Similarly, *in vivo* expression values relative to those for GP1 were, unsurprisingly, lower than those for hypha-defective strains due to intact *EFG1* signaling in the WT control. In any case, elevated forced SAP overexpression was indeed confirmed *in vivo* in our engineered strains.

FIG 1 Construction of hypha-defective *C. albicans* strains that overexpress *SAP2* or *SAP5*. (A) *C. albicans* strains were inoculated into RPMI medium and assessed for hypha formation by standard light microscopy. (i) GP1; (ii) GF54F+pWB3; (iii) GF54F+SAP2; (iv) GF54F+SAP5. Images are representative. (B) qPCR analysis of *SAP2* and *SAP5* gene expression in SAP overexpression strains compared to that in GF54F+pWB3 and normalized to the *ACT1* control gene ($\Delta\Delta C_t$ method). Data are reported as means and SEM ($n = 3$ independent repeats). TF1 and TF2 designations indicate independently created transformants. The dashed line indicates normalized fold expression in GF54F+pWB3. (C) BSA degradation assay depicting the protease activity (hazy zone of clearance) in GF54F+pWB3 compared to that in SAP overexpression strains. Two representative transformants (TF1 and TF2) are depicted per expression strain. Images are representative of 3 independent repeats.

In order to rule out the possibility that 5-fluoroorotic acid (5-FOA) selection of GF54F impaired our hypha-defective strain from expressing SAPs via an *EFG1*-independent mechanism, we constructed an *EFG1* revertant strain (*EFG1*-Rev) (Fig. S2). Restoration of *EFG1* restored the capacity to form hypha during growth in RPMI medium *in vitro* and at the vaginal mucosa *in vivo* (Fig. S2). Importantly, reintegration of *EFG1* rescued expression of *SAP2* and *SAP5* *in vivo*, to levels comparable to WT levels (Fig. S2). Thus,

FIG 2 Strains of *Candida* engineered to overexpress *SAP2* or *SAP5* in an *efg1Δ/Δ cph1Δ/Δ* background remain in the yeast form and selectively express SAPs *in vivo*. Vaginal lavage fluids were obtained at day 3 postinoculation and stained by the Papanicolaou technique, and images were captured by standard light microscopy. Green arrows depict the following *C. albicans* strains: GP1 (A), GF54F+pWB3 (B), GF54F+SAP2 (C), and GF54F+SAP5 (D). Images are representative of each group. qPCR data on *SAP2* and *SAP5* gene expression in SAP overexpression strains recovered from vaginal lavage fluid *in vivo* were compared to those for GF54F+pWB3 (E) or WT strain GP1 (F) and normalized to the *ACT1* control gene ($\Delta\Delta C_T$ method). Data are reported as means and SEM ($n = 4$ mice per group).

loss of *EFG1* signaling in the hypha-defective strain GF54F is likely responsible for reduced SAP expression *in vivo*.

Given that we had previously observed a defect in murine VVC immunopathology by using a *sap5Δ/Δ* strain, we expected that overexpression of *SAP2* or *SAP5* may restore virulence and/or the capacity to cause damage to an otherwise avirulent hypha-defective strain (4, 6). Intravaginal challenge with WT strain GP1, GF54F+pWB3 (empty vector), GF54F+SAP2, or GF54F+SAP5 demonstrated that CFU levels were significantly elevated for all yeast-dominant strains compared to those for the robust hypha-forming strain GP1 (Fig. 3A). These data are consistent with our previous observations using hypha-defective strains HLC52, HLC54, and TNRG1 (*NRG1* overexpressor) and are likely due to a lack of immune activation, increased cell turnover at the vaginal mucosa, the plating efficiencies of yeast versus hyphal cells, or a combination of the above (4). Quantification of neutrophils in the vaginal lavage fluid at day 3 postinoculation (optimal induction of immunopathology, based on previous studies) revealed that all hypha-defective variants, including those overexpressing *SAP2* or *SAP5*, failed to recruit polymorphonuclear leukocytes (PMNs) to levels similar to those for infection with the hypha-competent WT control (Fig. 3B). Surprisingly, SAP overexpression strains did not recruit more neutrophils than their isogenic empty vector control did. We previously identified the pleiotropic innate inflammatory cytokine IL-1 β as a relevant biomarker of vaginal immunopathology and known effector of inflammasome activa-

FIG 3 Overexpression of *SAP2* or *SAP5* in the hypha-defective strain GF54F does not induce immunopathology during murine vaginitis. Vaginal lavage fluids obtained from mice inoculated with GP1, GF54F+pWB3, GF54F+SAP2, or GF54F+SAP5 were analyzed at day 3 postinoculation for fungal burden (medians) (A), PMN recruitment as determined by microscopy (means and SEM) (B), IL-1 β secretion as determined by ELISA (means and SEM) (C), and LDH release as determined by enzymatic assay (means and SEM) (D). All data are cumulative from two independent experiments using 4 mice per group. Statistical testing of significance was achieved by using one-way ANOVA with the Kruskal-Wallis (CFU data) or Tukey's (all others) posttest, and significance is denoted as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 8$ mice per group).

tion, a crucial host signaling pathway activated during murine VVC (4, 6, 22–25). Importantly, recombinant SAPs have also been demonstrated to activate the inflammasome and to liberate IL-1 β in the vaginal epithelium (16, 26, 27). However, overexpression of either *SAP2* or *SAP5* failed to elicit IL-1 β in the vaginal lavage fluid of mice, and the IL-1 β levels were similar to that of the isogenic empty vector control; IL-1 β levels were significantly reduced compared to those for infection with GP1 (Fig. 3C). SAP activity has previously been linked to mucosal damage, so LDH release was assessed in the vaginal lavage fluid of mice challenged with the above strains. Similar to results obtained for PMN recruitment and IL-1 β release, LDH was significantly reduced in all hypha-defective strains compared to that of the WT and was not increased in hypha-defective strains engineered to overexpress *SAP2* or *SAP5* compared to the isogenic empty vector control level (Fig. 3D). Similarly, complementation of *EFG1* also led to colonization, recruitment of neutrophils to the vaginal lumen, IL-1 β release (data not shown), and LDH release (data not shown) similar to those of strain GP1 (Fig. S2).

These results were surprisingly disparate from those reported in the literature, in which recombinant SAPs alone were reported to induce murine vaginitis immunopathology, including robust neutrophil recruitment and IL-1 β release at the vaginal mucosa (16, 26, 27). Derivatives of strain GF54F are indeed deleted for *EFG1* and *CPH1*, but they also contain partial deletions of the iron-related transcription factor gene *IRO1* (a leftover from initial deletion of *URA3* in its parental strain) and a *URA3* gene reinserted at the nonnative *RPS10* locus (28). Therefore, we hypothesized that perhaps these multiple genetic defects synergize to significantly impair fungal fitness in the

murine vagina or that GF54F contains nontargeted mutations that similarly impair the capacity to elicit immunopathology. Therefore, we reconstructed SAP-overexpressing strains by using a pLUX-based vector originally described by Ramon and Fonzi, in which a single copy of *IRO1* is restored and *URA3* is integrated at its native locus (29). In addition, an independently constructed *efg1Δ/Δ cph1Δ/Δ* strain (termed CEF2) was obtained and subsequently used for SAP overexpression (30). Resultant CEF2-based transformants were verified for SAP overexpression by qPCR and functional assay, with results comparable to those for GF54F-based strains, and reintegration of CEF2 with an *EFG1* allele restored hypha formation and immunopathogenic capacity (data not shown). Similar to the findings with GF54F, overexpression of *SAP2* or *SAP5* failed to induce robust immunopathology over that of the empty vector control (CEF2+pKE4), yet all hypha-defective strains induced significantly less neutrophil recruitment (Fig. 4B), IL-1 β (Fig. 4C), and LDH (not shown) than those for infection with the WT strain GP1. Again, similar to our previous observations, fungal burdens were significantly higher after challenge with hypha-defective strains (Fig. 4A). Taken together, these observations also suggest that a gain-of-function *IRO1* or native *URA3* locus does not enhance virulence or colonization at the vaginal mucosa in the context of *EFG1* and *CPH1* deletion. Other reports of SAP-induced vaginal neutrophil recruitment have utilized flow cytometry to quantify PMNs in vaginal lavage fluid following *Candida* challenge (26, 27). In order to rule out any potential difference or bias between methodologies, cells from the vaginal lavage fluid were stained with fluorescently labeled antibodies specific for the murine cell surface neutrophil markers Ly6G and CD11b. Flow cytometry data were in excellent agreement with those obtained by staining and quantitative microscopy, in which SAP overexpression in hypha-defective strains failed to induce robust neutrophil migration into the vaginal lumen (Fig. 4D). Again, neutrophil recruitment in all hypha-defective strains was significantly reduced compared to that for challenge with strain GP1 (Fig. 4D), as indicated in representative flow cytometry plots (Fig. 4E and F).

Collectively, these results demonstrate that overexpression of the archetypical yeast- and hypha-associated proteinase genes *SAP2* and *SAP5* in a largely hypha-defective *efg1Δ/Δ cph1Δ/Δ* background fails to induce WT levels of immunopathology. Although deletion of various SAPs (these two included) seemingly diminishes the capacity to damage host tissue and elicit innate mucosal immune responses, it appears that hyphae are necessary to potentiate this activity. It is possible that cells of the keratinized epithelia are unresponsive to SAP-mediated degradation and mask underlying susceptible mucosal layers that can only be reached by invading hypha filaments or that formation of a hypha-induced epithelial "invasion pocket" creates microniches of high SAP concentration sufficient for immune activation (31). It is also possible that the SAPs synergize with other hypha-associated virulence factors to elicit robust immunopathology and are by themselves insufficient at biologically relevant concentrations. For example, the recently described hypha-specific fungal toxin candidalysin (product of the *ECE1* gene), which presumably intercalates into cellular membranes, may reach its target more effectively once SAPs have degraded host extracellular matrix proteins. Similarly, fungal adhesins may have better access to or increased binding kinetics for SAP-modified host tissue strata.

These results are somewhat unexpected given that several reports have demonstrated that introduction of recombinantly produced SAPs (namely, Sap2p and Sap6p) into the murine vaginal lumen elicits neutrophil recruitment to levels observed during challenge with WT *C. albicans* (26, 27). It is possible that differences between these studies and the results reported herein are due to concentration-dependent effects, as a bolus dose of recombinant SAP may be sufficient to initiate an inflammatory event. Although we could not explicitly measure secreted SAP, we typically observe targeted protein expression levels of ~ 0.1 $\mu\text{g/ml}$ in the culture supernatant by using similar *TEF1*-based overexpression strategies (B. Peters, unpublished observation). Studies with recombinant SAPs used an intravaginal inoculum of 0.5 μg , a dose that is expectedly supraphysiologic and likely higher than what strains constructed in this study would

FIG 4 Overexpression of SAP2 or SAP5 in the hypha-defective strain CEF2 similarly does not induce vaginal immunopathology. Vaginal lavage fluids obtained from mice inoculated with GP1, CEF2+pKE4, CEF2+SAP2, or CEF2+SAP5 were analyzed at day 3 postinoculation for fungal burden (medians) (A), PMN recruitment as determined by microscopy (means and SEM) (B), and IL-1 β secretion as determined by ELISA (means and SEM) (C). (D) Flow cytometry analysis of Ly6G⁺ CD11b⁺ cells was also undertaken. Quantitative data obtained by flow cytometry are presented as percentages of Ly6G⁺ CD11b⁺ cells in the lavage fluid (means and SEM). Representative scatterplots are shown for infection with GP1 (E) and CEF2+pKE4 (F), demarcating the PMN gate (R6) and double-positive populations. Statistical testing of significance was achieved by using one-way ANOVA with the Kruskal-Wallis (CFU data) or Tukey's (all others) posttest, and significance is denoted as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 10$ mice per group).

TABLE 1 Strains used or constructed for this study

Strain	Parent	Genotype	Reference
BWP17	CAI4	<i>ura3Δ/Δ iro1Δ/Δ his1Δ/Δ arg4Δ/Δ</i>	37
GP1	BWP17	<i>ura3Δ/ura3Δ-URA3-IRO1 iro1Δ/iro1Δ his1Δ/his1Δ:HIS1 arg4Δ/arg4Δ:ARG4</i>	This study
HLC54	CAI4	<i>ura3Δ/Δ cph1Δ/Δ efg1Δ/efg1-URA3</i>	19
GF54F	HLC54	<i>ura3Δ/Δ cph1Δ/Δ efg1Δ/Δ</i>	This study
<i>efg1Δ/Δ cph1Δ/Δ</i> mutant	JKC19	<i>ura3Δ/Δ cph1Δ/Δ efg1Δ/efg-URA3</i>	30
CEF2	<i>efg1Δ/Δ cph1Δ/Δ</i> mutant	<i>ura3Δ/Δ cph1Δ/Δ efg1Δ/Δ</i>	This study
GF54F+pWB3	GF54F	<i>ura3Δ/Δ cph1Δ/Δ efg1Δ/Δ RP10::URA3-TEF1pr</i>	This study
GF54F+SAP2	GF54F	<i>ura3Δ/Δ cph1Δ/Δ efg1Δ/Δ RP10::URA3-TEF1pr-SAP2</i>	This study
GF54F+SAP5	GF54F	<i>ura3Δ/Δ cph1Δ/Δ efg1Δ/Δ RP10::URA3-TEF1pr-SAP5</i>	This study
CEF2+pKE4	CEF2	<i>ura3Δ/ura3Δ-URA3-IRO1-TEF1pr cph1/cph1 efg1/efg1</i>	This study
CEF2+SAP2	CEF2	<i>ura3Δ/ura3Δ-URA3-IRO1-TEF1pr-SAP2 cph1/cph1 efg1/efg1</i>	This study
CEF2+SAP5	CEF2	<i>ura3Δ/ura3Δ-URA3-IRO1-TEF1pr-SAP5 cph1/cph1 efg1/efg1</i>	This study
EFG1-Rev	GF54F	<i>ura3Δ/Δ cph1Δ/Δ efg1Δ/efg1Δ-URA3-EFG1pr-EFG1</i>	This study

secrete. That said, since SAP expression in these overexpression constructs is constitutive, there should be a sustained release over the infectious time course that may very well surpass bolus delivery. Another discrepancy between the design of our study and recombinant SAP studies involves selection of the mouse strain background. We used inbred C57BL/6 mice that are highly sensitive to estrogen administration, including robust keratinization of the vaginal epithelium, while studies by Gabrielli et al. and Pericolini et al. (26, 27) used outbred CD-1 mice, which are known to be highly resistant to estrogenic modulation (32). Thus, because of estrogen resistance in CD-1 mice, it is likely that a greater number of nonkeratinized epithelial cells are exposed to SAP in these mice and that discordant immune profiles in these distinct cell subtypes can partially explain discrepant results. However, a failure to completely achieve pseudoeutrus (e.g., using the CD-1 strain) poses difficulty in quantification of *Candida*-induced neutrophil recruitment, as mice will progress approximately every 48 h to the diestrus phase, in which neutrophils migrate in large quantities into the vaginal lumen to eliminate the corpus luteum (33).

Overall, these results begin to unravel the complex virulence factor regulation associated with the yeast-to-hypha switch at the vaginal mucosa and demonstrate that overexpression of *SAP2* or *SAP5* in a predominantly blastoconidial morphotype is insufficient to induce robust vaginitis immunopathology. These results also begin to explain how clinical isolates of *C. albicans* may asymptotically colonize the vagina, despite retaining the capacity to secrete SAPs even during blastoconidial growth. Moreover, the overexpression strategies outlined herein will be useful for additional studies to decouple activity of hypha-associated factors from physical morphological transition.

MATERIALS AND METHODS

Ethics statement. The animals used in this study were housed in AAALAC-approved facilities located in the Regional Biocontainment Laboratory (RBL) at the University of Tennessee Health Sciences Center (UTHSC). The UTHSC Animal Care and Use Committee approved all animals and protocols. Mice were given standard rodent chow and water *ad libitum*. Mice were monitored for signs of distress, including noticeable weight loss and lethargy.

Microorganism growth. *C. albicans* strains were maintained as glycerol stocks stored at -80°C . A small amount of stock was spread onto yeast extract-peptone-dextrose (YPD) agar and incubated at 30°C for 48 h to obtain isolated colonies. A single colony was transferred to 10 ml of YPD and incubated at 30°C with shaking at 200 rpm for 18 h prior to vaginal infection.

Strains and primers. All strains used or generated for construction of overexpression strains and controls can be found in Table 1. All primers used for strain construction or quantitative PCR (qPCR) are listed in Table S1 in the supplemental material.

Vector construction. We took two different genetic approaches to construct SAP-overexpressing strains. First, the *TEF1* promoter and multiple-cloning site of plasmid pKE4 was PCR amplified using primers PRTEF1AMPF-KpnI and PKE4MCSAMPR-NheI to generate an amplicon containing terminal KpnI and NheI restriction sites (Fig. S1) (34, 35). The expression plasmid ClpACT-CYC was digested with KpnI and NheI to remove the *ACT1* promoter through sequences just upstream of the *Saccharomyces cerevisiae* CYC terminator sequence (ScCYCt) (36). After digestion with KpnI and NheI, the pKE4 amplicon was ligated into digested ClpACT-CYC to produce the expression vector pWB3. After transformation and propagation in *Escherichia coli* DH5 α , pWB3 was digested with Sall and MluI. The *SAP2* and *SAP5* ORFs were PCR amplified with primers SAP2ORFF-Sall and SAP5ORFR-MluI and primers SAP5ORFF-Sall and

SAP5ORFR-MluI, respectively. SAP amplicons were digested with Sall and MluI and ligated into cut pWB3 to yield SAP expression constructs pWB3-SAP2 and pWB3-SAP5. In order to construct an *EFG1* revertant strain, the *EFG1* ORF and ~2 kb of promoter sequence was amplified using primers EFG1AmpF-Apal and EFG1ORFR-MluI, digested with the corresponding enzymes, and ligated into the Apal- and MluI-digested CIP10ACT-CYC vector. All plasmids were verified for correct sequence by Sanger methodology (Molecular Resource Center, UTHSC), using primers PRTEF1SEQF and RP10SEQR.

A second distinct yet very similar approach was taken for transformation of CEF2. However, instead of using vector pWB3, *SAP2/5* ORF fragments were cloned directly into plasmid pKE4 to generate expression vectors pKE4-SAP2 and pKE4-SAP5. Notable differences in pKE4 include an *ADH3* terminator sequence, a full-length *IRO1* locus, and targeted integration at the native *IRO1-URA3* loci. All steps were essentially similar to those described above, except that the plasmid sequence was verified by PCR using primers PRTEF1SEQF and ADH13SEQR.

Strain construction. The prototrophic control strain GP1 was constructed through the sequential transformation of strain BWP17 (*ura3Δ/Δ arg4Δ/Δ his1Δ/Δ*) with NruI-cut pGEMHIS1, ClaI-cut pRSARG4ΔSpe, and NheI-cut pLUX (29, 37). Full restoration of the *HIS1*, *ARG4*, and *URA3-IRO1* loci was then confirmed using primer pairs HIS1DETF9-HIS1DETR5, ARG4DETF7-ARG4DETR7, and LUXINTDETF-LUXINTDETR, respectively.

The well-characterized *C. albicans* strain HLC54 (a homozygous deletion mutant lacking both the *EFG1* and *CPH1* transcription factors) was cultured on yeast nitrogen base (YNB) medium containing 5-fluoroorotic acid (5-FOA) (1 mg/ml) and uridine (0.05 mg/ml) to select for *ura3Δ/Δ* mutants (19). Loss of *URA3* was confirmed by PCR amplification of genomic DNA by use of primers internal to the *URA3* open reading frame (URA3DETF and URA3DETR) and by an inability to grow on YNB lacking uridine. This strain was named GF54F. In a similar approach, an independently constructed *efg1Δ/Δ cph1Δ/Δ* strain was obtained as described previously and underwent 5-FOA selection to generate a *ura3Δ/Δ* mutant termed CEF2 (30). Retention of the genomic *EFG1* and *CPH1* deletions was confirmed in both GF54F and CEF2 by PCRs using primers EFG1DETF and EFG1DETR and primers CPH1DETF and CPH1DETR, respectively.

Vectors pWB3, pWB3-SAP2, and pWB3-SAP5 were digested with StuI to target integration at the *RPS10* locus and then transformed into GF54F by the lithium acetate method, and transformants were selected on YNB lacking uridine to yield strains GF54F+pWB3, GF54F+SAP2, and GF54F+SAP5 (Fig. S1) (38). Similarly, vector CIP10-EFG1 was digested with PstI to integrate *EFG1* at its native locus to yield strain EFG1-Rev. Transformation was confirmed by PCR amplification of *C. albicans* genomic DNA by using primers PRTEF1SEQF plus RP10SEQR or EFG1PrDETF plus RP10SEQR and EFG1DETF plus EFG1DETR. Similarly, vectors pKE4, pKE4-SAP2, and pKE4-SAP5 were digested with NheI to target integration at the *IRO1-URA3* loci and then transformed into CEF2 by the lithium acetate method to yield strains CEF2+pKE4, CEF2+SAP2, and CEF2+SAP5. Correct integration was verified by PCR amplification of genomic DNA with primers LUXINTF plus LUXINTR and PRTEF1SEQF plus SAP2ORFR or SAP5ORFR.

Hyphal growth assay. A single colony of each *C. albicans* strain was transferred to buffered RPMI 1640 medium and incubated at 37°C overnight with shaking at 200 rpm. The following day, 10 μ l of cells was transferred to a glass slide and examined by standard light microscopy, and images were captured using a Nikon Ni-U microscope with the NIS elements software package.

RNA extraction. *C. albicans* was cultivated in liquid YPD medium overnight at 30°C in a shaking incubator (200 rpm). The following day, cells were diluted 1:100 in fresh YPD and grown for 3 h at 30°C with shaking. *C. albicans* cells were also isolated from vaginal lavage fluid to assess *in vivo* gene expression (see the lavage procedure below). RNA was extracted by the hot acid-phenol method as previously described (39). Aqueous-phase RNA was precipitated with 3 M sodium acetate-ethanol, followed by brief washes in 70% ethanol. RNA pellets were air dried and resuspended in sterile water. The integrity of RNA (1 μ l) was assessed by morpholinepropanesulfonic acid (MOPS) gel electrophoresis and visualization of intact 18S and 28S bands. RNA concentrations were assessed by using a NanoDrop spectrophotometer to measure $A_{260}/_{280}$.

qPCR for SAP gene expression. Extracted RNA concentrations were equalized among samples, and 200-ng aliquots were treated with RNase-free DNase according to the manufacturer's instructions (Thermo Fisher). RNA was reverse transcribed using random hexamers and a RevertAid kit according to the manufacturer's protocol (Thermo Fisher). Forward and reverse primers (final concentration, 0.5 μ M) specific for the *SAP2*, *SAP5*, or *ACT1* ORF were used in conjunction with 2 $\times$ Maxima SYBR green according to the manufacturer's instructions (Thermo Fisher) to amplify 100-bp fragments from approximately 20 ng of cDNA. qPCRs were monitored and analyzed with an Applied Biosystems 7500 platform and software. Expression levels of *SAP2* and *SAP5* in overexpression strains were compared to those of a reference gene (*ACT1*) and those in GF54F+pWB3 or GP1 by using the $\Delta\Delta C_T$ method (where C_T is the threshold cycle) (40). Additionally, qPCR experiments using similar methodology were conducted to compare SAP gene expression levels in the WT and EFG1-Rev strains.

SAP functional assay. *C. albicans* was grown overnight in YPD as described above. The following day, 5 μ l of culture was spotted onto yeast extract-bovine serum albumin agar plates (YE-BSA; 1.17% yeast carbon base, 0.01% yeast extract, 0.1% BSA) (20). Plates were statically incubated for 3 days at 30°C, and photographs were captured using a G-Box platform (Syngene).

Murine model of vaginal candidiasis. The murine model of *Candida* vaginitis has been reported extensively in the literature and was performed as described previously (4, 6). Female 6- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories and housed in isolator cages mounted on ventilated racks. Mice were administered 0.1 mg of estrogen (β -estradiol 17-valerate; Sigma) dissolved in 0.1 ml of sesame oil subcutaneously 72 h prior to inoculation with *C. albicans*. Stationary-phase cultures of *C. albicans* isolates were washed three times in sterile, endotoxin-free phosphate-buffered saline (PBS)

and resuspended in a 0.2× volume of PBS. Cell suspensions were diluted, counted on a Neubauer hemocytometer, and adjusted to 5×10^8 CFU/ml in sterile PBS. Estrogen-treated mice were intravaginally inoculated with 10 μ l of the standardized blastoconidial cell suspension, generating an inoculum size of 5×10^6 blastoconidia. Mice underwent vaginal lavage with 100 μ l of PBS upon sacrifice. Resultant lavage fluids were spiked with 1 μ l of 100× EDTA-free protease inhibitors (cOmplete; Roche) and kept on ice until processing for immunopathological markers. All animal experiments were conducted in duplicate, and the resulting data were combined.

Assessment of fungal burdens and vaginitis immunopathology. All immunopathological markers were assessed as described previously (4). (i) Lavage fluid was serially diluted 10-fold by using the drop-plate method and plated onto YPD agar containing 50 μ g/ml chloramphenicol, plates were incubated for 24 h at 37°C, and the resulting colonies were enumerated. Numbers of CFU per milliliter per group are reported as medians. (ii) Lavage fluid (10 μ l) was smeared onto glass slides and stained by the Papanicolaou technique to assess polymorphonuclear leukocyte (PMN) recruitment (small, blue cells with multilobed nuclei). PMNs were counted in 5 nonadjacent fields by standard light microscopy, using a 40× objective, and values are reported as means $\pm$ standard errors of the means (SEM). (iii) Murine IL-1 β was assessed in clarified, diluted (1:20) vaginal lavage fluid by using a commercial enzyme-linked immunosorbent assay (ELISA; eBioscience) according to the manufacturer's protocol. Results are reported as means $\pm$ SEM. (iv) Lactate dehydrogenase (LDH) activity was measured in clarified, diluted (1:100) lavage fluid by using the commercially available CytoTox 96 nonradioactive cytotoxicity assay (Promega). Results are reported as means $\pm$ SEM.

Flow cytometry. Vaginal lavage fluid was clarified by centrifugation at 3,000 rpm for 3 min. The resulting cell pellet was washed in ice-cold PBS and adjusted to approximately 5×10^6 cells/ml in fluorescence-activated cell sorter (FACS) buffer (eBioscience) after counting on a hemacytometer. Aliquots (100 μ l) of each cell suspension were transferred to FACS tubes and blocked with anti-CD16/32 to inhibit surface Fc receptors per the manufacturer's instructions (eBioscience). After washing in FACS buffer, cells were incubated with antibodies diluted to 0.5 μ g/ml in FACS buffer and stained for 30 min at room temperature protected from light. All antibodies were purchased from eBioscience and were as follows: isotype control, fluorescein isothiocyanate (FITC)-anti-rat IgG2b, clone RTK4530; isotype control, allophycocyanin (APC)-anti-rat IgG2b, clone RTK4530; FITC-anti-mouse Ly6G, clone 1A8; and APC-anti-mouse CD11b, clone M1/70. Cells were washed twice in FACS buffer and analyzed on a Novocyte 3000 flow cytometer, using FITC/APC filter sets and NovoExpress software. Singly stained and isotype-stained controls were conducted to establish the neutrophil marker gate (data not shown). Data are expressed as mean percentages of Ly6G⁺ CD11b⁺ cells in the gated population $\pm$ SEM.

Statistical analyses. All experiments were conducted using groups of mice ($n = 4$) and repeated in duplicate as determined by power analyses. All data were plotted and analyzed for statistical significance by using GraphPad Prism software. Data sets were tested for normality by using the Shapiro-Wilks test. Data were compared by one-way analysis of variance (ANOVA) and Tukey's (parametric) or Kruskal-Wallis (nonparametric) posttest. Graphs in figures are annotated to denote significance levels.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00248-17>.

SUPPLEMENTAL FILE 1, PDF file, 2.2 MB.

ACKNOWLEDGMENTS

We kindly thank Gerry Fink (Whitehead Institute, Massachusetts Institute of Technology), Alexander Johnson (University of California, San Francisco), and Bill Fonzi (Georgetown University) for providing transcriptional regulator mutants or vectors used for construction of overexpression strains.

These studies were supported by NIH National Institute of Allergy and Infectious Diseases (NIAID) grants K22AI110541 (awarded to B.M.P.) and R01AI099080 (awarded to G.E.P.). The UTHSC College of Pharmacy Dean's Enhancement Program, awarded to B.M.P., also supported these studies.

All experimental design was conducted by B.M.P. and G.E.P. H.M.E.W., W.S.B., K.S.B., and J.L. performed all experimental techniques and data analysis and constructed all GF54F-based strains. G.E.P. constructed strain GP1 and CEF2-based strains. All authors aided in experimental critique and manuscript preparation.

REFERENCES

1. Sobel JD. 1997. Vaginitis. *N Engl J Med* 337:1896–1903. <https://doi.org/10.1056/NEJM199712253372607>.
2. Sobel JD. 2007. Vulvovaginal candidosis. *Lancet* 369:1961–1971. [https://doi.org/10.1016/S0140-6736\(07\)60917-9](https://doi.org/10.1016/S0140-6736(07)60917-9).
3. Fidel PL, Jr, Barousse M, Espinosa T, Ficarra M, Sturtevant J, Martin DH, Quayle AJ, Dunlap K. 2004. An intravaginal live *Candida* challenge in humans leads to new hypotheses for the immunopathogenesis of vulvovaginal candidiasis. *Infect Immun* 72:2939–2946. <https://doi.org/10.1128/IAI.72.5.2939-2946.2004>.
4. Peters BM, Palmer GE, Nash AK, Lilly EA, Fidel PL, Jr, Noverr MC. 2014.

- Fungal morphogenetic pathways are required for the hallmark inflammatory response during *Candida albicans* vaginitis. *Infect Immun* 82: 532–543. <https://doi.org/10.1128/IAI.01417-13>.
5. Kumamoto CA, Vences MD. 2005. Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. *Cell Microbiol* 7:1546–1554. <https://doi.org/10.1111/j.1462-5822.2005.00616.x>.
 6. Bruno VM, Shetty AC, Yano J, Fidel PL, Jr, Noverr MC, Peters BM. 2015. Transcriptomic analysis of vulvovaginal candidiasis identifies a role for the NLRP3 inflammasome. *mBio* 6:e00182-15. <https://doi.org/10.1128/mBio.00182-15>.
 7. Naglik JR, Moyes D, Makwana J, Kanzaria P, Tschlakai E, Weindl G, Tappuni AR, Rodgers CA, Woodman AJ, Challacombe SJ, Schaller M, Hube B. 2008. Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. *Microbiology* 154:3266–3280. <https://doi.org/10.1099/mic.0.2008/022293-0>.
 8. Villar CC, Kashleva H, Nobile CJ, Mitchell AP, Dongari-Bagtzoglou A. 2007. Mucosal tissue invasion by *Candida albicans* is associated with E-cadherin degradation, mediated by transcription factor Rim101p and protease Sap5p. *Infect Immun* 75:2126–2135. <https://doi.org/10.1128/IAI.00054-07>.
 9. Cassone A, Vecchiarelli A, Hube B. 2016. Aspartyl proteinases of eukaryotic microbial pathogens: from eating to heating. *PLoS Pathog* 12:e1005992. <https://doi.org/10.1371/journal.ppat.1005992>.
 10. Felk A, Kretschmar M, Albrecht A, Schaller M, Beinhauer S, Nichterlein T, Sanglard D, Korting HC, Schafer W, Hube B. 2002. *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. *Infect Immun* 70:3689–3700. <https://doi.org/10.1128/IAI.73.3.1828-1835.2005>.
 11. Taylor BN, Staib P, Binder A, Biesemeier A, Sehnal M, Rollinghoff M, Morschhauser J, Schroppel K. 2005. Profile of *Candida albicans*-secreted aspartic proteinase elicited during vaginal infection. *Infect Immun* 73: 1828–1835. <https://doi.org/10.1128/IAI.73.3.1828-1835.2005>.
 12. Cassone A, Boccanera M, Adriani D, Santoni G, De Bernardis F. 1995. Rats clearing a vaginal infection by *Candida albicans* acquire specific, antibody-mediated resistance to vaginal reinfection. *Infect Immun* 63: 2619–2624.
 13. Cassone A, De Bernardis F, Torosantucci A, Tacconelli E, Tumbarello M, Cauda R. 1999. In vitro and in vivo anticandidal activity of human immunodeficiency virus protease inhibitors. *J Infect Dis* 180:448–453. <https://doi.org/10.1086/314871>.
 14. De Bernardis F, Arancia S, Morelli L, Hube B, Sanglard D, Schafer W, Cassone A. 1999. Evidence that members of the secretory aspartyl proteinase gene family, in particular SAP2, are virulence factors for *Candida* vaginitis. *J Infect Dis* 179:201–208. <https://doi.org/10.1086/314546>.
 15. De Bernardis F, Santoni G, Boccanera M, Spreghini E, Adriani D, Morelli L, Cassone A. 2000. Local anticandidal immune responses in a rat model of vaginal infection by and protection against *Candida albicans*. *Infect Immun* 68:3297–3304. <https://doi.org/10.1128/IAI.68.6.3297-3304.2000>.
 16. Pietrella D, Pandey N, Gabrielli E, Pericolini E, Perito S, Kasper L, Bistoni F, Cassone A, Hube B, Vecchiarelli A. 2013. Secreted aspartic proteases of *Candida albicans* activate the NLRP3 inflammasome. *Eur J Immunol* 43:679–692. <https://doi.org/10.1002/eji.201242691>.
 17. Dieterich C, Schandar M, Noll M, Johannes FJ, Brunner H, Graeve T, Rupp S. 2002. In vitro reconstructed human epithelia reveal contributions of *Candida albicans* EFG1 and CPH1 to adhesion and invasion. *Microbiology* 148:497–506. <https://doi.org/10.1099/00221287-148-2-497>.
 18. Moyes DL, Murciano C, Runglall M, Islam A, Thavaraj S, Naglik JR. 2011. *Candida albicans* yeast and hyphae are discriminated by MAPK signaling in vaginal epithelial cells. *PLoS One* 6:e26580. <https://doi.org/10.1371/journal.pone.0026580>.
 19. Lo HJ, Kohler JR, DiDomenico B, Loebeberg D, Cacciapuoti A, Fink GR. 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* 90:939–949. [https://doi.org/10.1016/S0092-8674\(00\)80358-X](https://doi.org/10.1016/S0092-8674(00)80358-X).
 20. Bernardo SM, Khalique Z, Kot J, Jones JK, Lee SA. 2008. *Candida albicans* VPS1 contributes to protease secretion, filamentation, and biofilm formation. *Fungal Genet Biol* 45:861–877. <https://doi.org/10.1016/j.fgb.2008.01.001>.
 21. Korting HC, Hube B, Oberbauer S, Januschke E, Hamm G, Albrecht A, Borelli C, Schaller M. 2003. Reduced expression of the hyphal-independent *Candida albicans* proteinase genes SAP1 and SAP3 in the efg1 mutant is associated with attenuated virulence during infection of oral epithelium. *J Med Microbiol* 52:623–632. <https://doi.org/10.1099/jmm.0.05125-0>.
 22. Borghi M, De Luca A, Puccetti M, Jaeger M, Mencacci A, Oikonomou V, Pariano M, Garlanda C, Moretti S, Bartoli A, Sobel J, van de Veerdonk FL, Dinarello CA, Netea MG, Romani L. 2015. Pathogenic NLRP3 inflammasome activity during *Candida* infection is negatively regulated by IL-22 via activation of NLR4 and IL-1Ra. *Cell Host Microbe* 18:198–209. <https://doi.org/10.1016/j.chom.2015.07.004>.
 23. Jaeger M, Carvalho A, Cunha C, Plantinga TS, van de Veerdonk F, Puccetti M, Galosi C, Joosten LA, Dupont B, Kullberg BJ, Sobel JD, Romani L, Netea MG. 2016. Association of a variable number tandem repeat in the NLRP3 gene in women with susceptibility to RVVC. *Eur J Clin Microbiol Infect Dis* 35:797–801. <https://doi.org/10.1007/s10096-016-2600-5>.
 24. Nash EE, Peters BM, Lilly EA, Noverr MC, Fidel PL, Jr. 2016. A murine model of *Candida glabrata* vaginitis shows no evidence of an inflammatory immunopathogenic response. *PLoS One* 11:e0147969. <https://doi.org/10.1371/journal.pone.0147969>.
 25. Yano J, Kolls JK, Happel KI, Wormley F, Wozniak KL, Fidel PL, Jr. 2012. The acute neutrophil response mediated by S100 alarmins during vaginal *Candida* infections is independent of the Th17-pathway. *PLoS One* 7:e46311. <https://doi.org/10.1371/journal.pone.0046311>.
 26. Gabrielli E, Sabbatini S, Roselletti E, Kasper L, Perito S, Hube B, Cassone A, Vecchiarelli A, Pericolini E. 2016. In vivo induction of neutrophil chemotaxis by secretory aspartyl proteinases of *Candida albicans*. *Virulence* 7:819–825. <https://doi.org/10.1080/21505594.2016.1184385>.
 27. Pericolini E, Gabrielli E, Amacker M, Kasper L, Roselletti E, Luciano E, Sabbatini S, Kaeser M, Moser C, Hube B, Vecchiarelli A, Cassone A. 2015. Secretory aspartyl proteinases cause vaginitis and can mediate vaginitis caused by *Candida albicans* in mice. *mBio* 6:e00724-15. <https://doi.org/10.1128/mBio.00724-15>.
 28. Chibana H, Uno J, Cho T, Mikami Y. 2005. Mutation in *IRO1* tightly linked with *URA3* gene reduces virulence of *Candida albicans*. *Microbiol Immunol* 49:937–939. <https://doi.org/10.1111/j.1348-0421.2005.tb03686.x>.
 29. Ramon AM, Fonzi WA. 2003. Diverged binding specificity of Rim101p, the *Candida albicans* ortholog of PacC. *Eukaryot Cell* 2:718–728. <https://doi.org/10.1128/EC.2.4.718-728.2003>.
 30. Braun BR, Johnson AD. 2000. *TUP1*, *CPH1* and *EFG1* make independent contributions to filamentation in *Candida albicans*. *Genetics* 155:57–67.
 31. Moyes DL, Wilson D, Richardson JP, Mogavero S, Tang SX, Wernecke J, Hofs S, Gratacap RL, Robbins J, Runglall M, Murciano C, Blagojevic M, Thavaraj S, Forster TM, Hebecker B, Kasper L, Vizcay G, Iancu SI, Kichik N, Hader A, Kurzai O, Luo T, Kruger T, Kniemeyer O, Cota E, Bader O, Wheeler RT, Gutschmann T, Hube B, Naglik JR. 2016. Candidalysin is a fungal peptide toxin critical for mucosal infection. *Nature* 532:64–68. <https://doi.org/10.1038/nature17625>.
 32. Clemons KV, Spearow JL, Parmar R, Espiritu M, Stevens DA. 2004. Genetic susceptibility of mice to *Candida albicans* vaginitis correlates with host estrogen sensitivity. *Infect Immun* 72:4878–4880. <https://doi.org/10.1128/IAI.72.8.4878-4880.2004>.
 33. Byers SL, Wiles MV, Dunn SL, Taft RA. 2012. Mouse estrous cycle identification tool and images. *PLoS One* 7:e35538. <https://doi.org/10.1371/journal.pone.0035538>.
 34. Johnston DA, Tapia AL, Eberle KE, Palmer GE. 2013. Three prevacuolar compartment Rab GTPases impact *Candida albicans* hyphal growth. *Eukaryot Cell* 12:1039–1050. <https://doi.org/10.1128/EC.00359-12>.
 35. Peters BM, Luna-Tapia A, Tournu H, Rybak JM, Rogers PD, Palmer GE. 2017. An azole-tolerant endosomal trafficking mutant of *Candida albicans* is susceptible to azole treatment in a mouse model of vaginal candidiasis. *Antimicrob Agents Chemother* 61:e00084-17. <https://doi.org/10.1128/AAC.00084-17>.
 36. Blackwell C, Russell CL, Argimon S, Brown AJ, Brown JD. 2003. Protein A-tagging for purification of native macromolecular complexes from *Candida albicans*. *Yeast* 20:1235–1241. <https://doi.org/10.1002/yea.1036>.
 37. Wilson RB, Davis D, Mitchell AP. 1999. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J Bacteriol* 181:1868–1874.
 38. Gietz RD, Woods RA. 2006. Yeast transformation by the LiAc/SS carrier DNA/PEG method. *Methods Mol Biol* 313:107–120.
 39. Collart MA, Oliviero S. 2001. Preparation of yeast RNA. *Curr Protoc Mol Biol* Chapter 13:Unit13.12. <https://doi.org/10.1002/0471142727.mb1312s23>.
 40. Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45. <https://doi.org/10.1093/nar/29.9.e45>.