

Original Article

The rod cell, a small form of *Candida albicans*, possesses superior fitness to the host gut and adaptation to commensalism

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Received 25 December 2023 Accepted 6 February 2024

Abstract

Candida albicans deploys various morphological forms through complex switching mechanisms, ensuring its survival and thriving as a commensal or pathogen in vastly different human niches. In this study, we demonstrate that a novel “rod” morphological form of *C. albicans* coexists and is interchangeable with previously reported white, gray, and opaque forms, constituting a tetra-stable phenotypic switching system. Rod cells arise from the *efg1* mutant of SC5314 cells or from the clinical BJ1097 strain cultured under glucose-free conditions. They are characterized by a distinct gene expression profile and can be stably maintained through *in vitro* passaging or *in vivo* inhabitation of the gastrointestinal (GI) tract of mice. Remarkably, the majority of the *efg1* mutant cells become rod cells in N-acetylglucosamine (GlcNAc)-containing medium, and the GlcNAc sensor Ngs1 is instrumental in converting the white or gray cells to the rod cells. Conversely, glucose inhibits rod cells through Cph1; consequently, the loss of Cph1 in the *efg1* mutant cells permits their conversion to rod cells in glucose-replete media. Notably, rod cells of the *efg1/cph1* mutant display superior adaptation and longer persistence in the murine GI environment than wild-type white cells. Taken together, these findings establish rod cells as a previously unappreciated form that is not only morphologically and transcriptionally distinguishable but also defined by specific genetic and environmental determinants, shedding light on complex fungus-host interactions.

Key words rod cell, phenotypic switching, Efg1, gut commensalism

Introduction

Candida albicans is a commensal resident in the gut of healthy humans but can become pathogenic in immunocompromised individuals. Central to its transition from commensalism to pathogenesis is the transition between various morphological forms, including white-opaque switching, white-GUT transition, and white-gray-opaque switching [1–5]. The white, gray, and opaque phenotypes manifest unique cellular and colonial morphologies. White cells are characterized by their relatively small volumes and round shapes, forming hemispherical colonies with a white coloration. Opaque cells display an elongated morphology

and larger sizes, resulting in flatter, rough, and dark-colored colonies [1–6]. Gray cells are smaller than opaque cells, forming dark-colored colonies with a smooth surface [5]. The transcription factors Efg1 and Wor1 play pivotal roles in white-opaque switching [7–14]. Liang *et al.* [15] and Park *et al.* [16–18] reported that the loss of *EFG1* function activated white-to-gray phenotypic switching in both mating-type locus (*MTL*) heterozygous strains and *MTL* homozygous strains. Notably, hemizygosity and mutation of *EFG1* in clinical isolates license this phenotypic transition in *C. albicans* cells [15–18]. Deletion of *EFG1* and *WOR1* in the clinical *C. albicans* strain BJ1097 stabilizes cells in the gray phase [5]. We previously

reported an opaque-like phenotype in the *efg1/wor1* mutant regulated by ambient pH [19]. However, this opaque-like form was subsequently classified as a “gray” phenotype by Park *et al.* [16]. Intriguingly, the “gray cells” reported from different laboratories exhibit tremendous heterogeneity in cell size, varying from strain to strain and from cell to cell. In Huang’s laboratory, the clinical *C. albicans* isolate BJ1097 displayed a broad range of gray cell sizes in response to various growth conditions [5]. In Bennett’s laboratory, an “INT” cell state (intermediate) of *EFG1* heterozygous cells was reported [15]. Due to the phenotypical similarity between INT and gray states, they referred to the INT state as the gray state. In Soll’s laboratory, two subgroups of the gray phenotype including “tiny elongate” and “transition” were noted [18]. In our laboratory, we also observed two distinct types of gray cells differing in size (big and small) when cultured in different media under various growth conditions. It is unknown whether small-sized “gray” cells can be maintained in high purity under specific conditions and whether they have the capability to transition into other established cell types.

In this study, we designate the smaller cell state as the “rod” state, as these smaller cells exhibit phenotypic similarities to bacilli (rod-like bacteria) in both cell shape and size. We uncovered that the carbon source plays a pivotal role in the formation of rod cells, and we successfully obtained rod cells in a high purity form from *efg1*-null mutants under specific culture conditions. Our results demonstrated that the rod state represents a novel cell morphological phenotype of *C. albicans* that is heritable and sustainable through many generations of cell division. The global gene expression profile of rod cells is distinct from that of gray cells. We posit that the rod form, along with the white, gray, and opaque forms, constitutes a tetra-stable switching system in *C. albicans*.

The mammalian gastrointestinal (GI) tract is a complex and dynamic microenvironment that hosts a large number of microorganisms. As a commensal resident in the human gut, *C. albicans* cells employ diverse strategies to adapt to their surroundings. Indeed, *C. albicans* can undergo white-to-opaque switch in conditions mimicking the gut microenvironment [3,20,21], and *Wor1* overexpression triggers white-to-GUT switch, promoting *C. albicans* commensalism in the mouse gut [2]. During commensalism, the GUT and opaque phenotypes competitively outperform the white phenotype. Notably, the gray phenotype, primarily associated with *Efg1* deficiency, has a competitive advantage over the white phenotype [2,5,15]. Non-filamentous gut-evolved strains exhibit remarkable fitness within the GI tract of antibiotic-treated mice [22,23]. Lo *et al.* [7] previously deleted the *CPH1* gene in an *efg1* mutant and constructed a non-filamentous *C. albicans* mutant strain that is avirulent in a systemic infection model. Further verification is needed to determine whether the genuine rod cells, which have previously been mixed with gray cells in prior studies for a decade, possess the capability to colonize the gut.

Here, we report that *efg1/cph1* mutant cells can switch to the rod form and maintain the rod phenotype under various growth conditions as well as in the gut. Additionally, *efg1/cph1* rod cells outcompeted wild-type white cells in a mouse GI commensal competition experiment. Our results suggest that *C. albicans* may survive in the GI microenvironment by shrinking to a smaller size, mimicking a gut bacillus. These findings provide insights into how fungi adapt to the host GI microenvironment and have implications for complex host-microbe interactions within the GI tract.

Materials and Methods

Media and growth conditions

Various types of *C. albicans* cells were cultivated under specific conditions to observe distinct phenotypes. White cells were obtained at 37°C on SCD plates supplemented with synthetic complete glucose (dextrose) (0.17% Difco yeast nitrogen base without ammonium sulfate, 37 mM ammonium sulfate, auxotrophic supplements, 2% glucose and 2% agar, pH 4.5). The gray cells were cultivated at 25°C on YPD plates (1% yeast extract, 2% peptone, 2% glucose and 2% agar). Opaque cells were incubated at 25°C with 20% CO₂ on YPD plates, and rod-shaped cells were grown at 25°C on YPE (YP + 2% ethanol) or YPG (YP + 2% GlcNAc) plates. The different cell types were distributed onto plates supplemented with YP (1% yeast extract, 2% peptone, 2% agar) or YP supplemented with various carbon sources for subsequent investigations.

Plasmid and strain construction

The plasmids used in this study are listed in Supplementary Table S1. The *C. albicans* strains used in this study are listed in Supplementary Table S2. The primers used are listed in Supplementary Table S3. DNA sequences of *C. albicans* genes were obtained from the *Candida* Genome Database (<http://www.candidagenome.org>). SC5314 genomic DNA was used as a template for all PCR amplifications of *C. albicans* genes.

CZQ and CXY30 strains were produced as following. The disrupting vector pCPC48/49 carrying the *CmLEU2* or *CdHIS1* marker was used as a template to delete target genes via PCR-based homologous recombination, as previously described [24]. *LoxP-CmLEU2-LoxP* (PLP) and *LoxP-CdHIS1-LoxP* (PHP) were assembled with 5' and 3' fragments of target genes by the fusion PCR method. The wild-type (SN152) or *efg1* mutant (CXY9) was used to construct the *ngs1* (CZQ) or *efg1/ngs1* (CXY30) mutant.

CXY3 and CXY32 strains were generated by amplifying *HSP31p*-Camcherry from the p673-*HSP31p*-CamCherry plasmid using primers P673-PCR-F and P673-PCR-R. Subsequently, this fragment was transformed into *C. albicans* strains HLC67 or HLC69 and was integrated into the *RPS10* site, resulting in the generation of CXY3 and CXY32 strains, respectively.

For *MTL* homozygous screening, the CXY4, CXY9, CXY21, CXY33, and CXY35 strains were screened from the initial strains CXY3, CXY8, CZQ, CXY32 and CXY34, respectively, using a method described previously [25].

Morphology observation

C. albicans or *E. coli* cells on plates under various growth conditions were harvested and washed three times with ddH₂O. Subsequently, the cells were analyzed by microscopy, at least 500 cells from five randomly selected zones were captured for observation under each condition, and the percentage of rod-shaped cells was determined. These experiments were repeated at least three times. To determine the sizes of the different cell types, 20 cells of each state were selected, and the length-to-width ratio was measured using ImageJ (v1.53t; NIH, Bethesda, USA).

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

RNA from *C. albicans* was extracted using the thermos-phenol method, following a modified version of the protocol described

previously [26]. Briefly, *C. albicans* cells were collected and cleaned with ice distilled water. After centrifugation to pellet the cells, they were suspended in 500 μ L of TES buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS). Then, an equal volume of phenol was added to the cell suspension, mixed and incubated at 65°C for 30 min with periodic inversion (mixing) every 10 min. The samples were then cooled and centrifuged at the highest speed for 5 min to separate the phases. The upper water phase containing RNA was collected. An equal volume of chloroform was added to the collected water phase, mixed, incubated on ice for 5 min and centrifuged to collect the upper aqueous phase again. In the upper aqueous phase, ethanol was added to the collected aqueous phase to a final concentration of 100%. The mixture was centrifuged at high speed for 5 min to precipitate the RNA. The RNA pellet was washed three times with 75% ethanol. After washing, the RNA pellet was dried. A TIANscript RT kit (KR104; TIANGEN, Beijing, China) was used to convert the extracted RNA into cDNA. Then qRT-PCR was performed as described previously [27]. Briefly, real-time PCRs were carried out with qPCR SyGreen 2-Step Detect Lo-ROX (FP205-02; TIANGEN) on a Roche Light cycler 96 real-time PCR detection system with the following program: initial melting occurred at 94°C for 300 s, followed by 30 cycles of melting at 95°C for 10 s followed by annealing at 55°C for 20 s then extension at 72°C for 34 s with fluorescent measurements taken at the end of each extension. Melt curves (60°C–94°C) with continuous acquisition were generated to verify single product. Relative expression levels of target genes were quantitatively normalized against the expression of *ACT1* using $\Delta\Delta$ CT method. The sequences of the primers are shown in [Supplementary Table S3](#).

RNA-seq data analysis

Library construction and RNA-seq were carried out by Tsingke Biotechnology (Beijing, China). Qualified reads were mapped to the *C. albicans* reference genome (GCF_000182965.3_ASM18296v3_genomic). Transcriptomic analysis of gray and rod cells was performed as described in our previous report [28]. We applied DESeq2 to identify differentially expressed genes (DEGs) between rod and gray cells, and the significant genes were identified with adjusted *P* value < 0.05 and log₂ fold-change > 1. Gene Ontology (GO) enrichment analysis was performed on the detected DEGs using g:Profiler [29].

Mouse commensal and competitive fitness experiments

All animal experiments involving mice were conducted in accordance with ethical guidelines and approved by the Animal Care and Use Committee of the Shanghai Institute of Biochemistry and Cell Biology, Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences. Mice for the study were obtained from Shanghai Laboratory Animal Co., Ltd (SLAC, Chinese Academy of Sciences, Shanghai, China) and were housed in compliance with institutional Animal Care and Use Committee Guidelines. Auto-claved distilled water containing 2 mg/mL streptomycin and 0.9 mg/mL penicillin G was provided to all animals throughout the experiment.

In the commensalism experiments, *efg1* (CXY4, *MTLa/a*) mutant cells were cultured on YP plate, or *efg1/cph1* (CXY33, *MTLa/a*) mutant cells were cultured on YPD plate for 2 days to induce rod morphology. Subsequently, 1×10^8 rod cells were orally administered to 6- to 8-week-old BALB/c mice (*n* = 3 to 5 mice per group) treated with antibiotics. Fresh fecal pellets were collected and plated

on YP or YPD agar plates supplemented with antibiotics at 25°C for one day. The strain's morphologies (phenotypes) were tracked over a 20-day period.

The competitive fitness experiments were similar to the commensalism experiments but involved a 1:1 mixture of wild-type and *efg1/cph1* (CXY33, *MTLa/a*) mutant *C. albicans* strains, as previously described [2]. After the cells were recovered from mouse fecal pellets, they were cultured on YP plates at 25°C for one day. The relative abundance (genotype) was determined by RT-qPCR using strain-specific primers ([Supplementary Table S3](#)).

Statistical analysis

Data are presented as the mean $\pm$ standard deviation (SD) or standard error of the mean (SEM). Two-tailed unpaired Student's *t* test was performed to compare the differences between treated groups and paired controls. Statistical analyses were performed using GraphPad Prism 8.30 (GraphPad Software, La Jolla, USA). *P* < 0.05 indicated significant difference.

Results

The rod-gray-opaque-white tetra-stable switching system in *C. albicans*

C. albicans was proposed to have a tristable switching system enabling the transition between white, gray, and opaque phenotypes under specific culture media and growth conditions [5]. Hemizygosity or mutation of the *EFG1* gene in clinical *C. albicans* isolates results in white or opaque to gray transition [15–18]. Intriguingly, the sizes of gray cells reported from different laboratories were markedly different [5,15,18]. To better compare the sizes of gray cells, we examined the gray phenotype of *efg1* mutant cells under different media or growth conditions. When white cells of the *efg1* mutant were streaked onto YP plate and incubated in air at room temperature, we observed three cell types, namely, white, gray and small rod-shaped cells ([Figure 1A](#)). Considering that small rod-shaped cells share a phenotypic resemblance with bacterial *Bacillus* cells in both shape and size, we henceforth designated these cells the “rod” cell state. To analyze white-gray-rod switching, we investigated the effects of carbon sources on rod cell formation. White cells of *efg1* mutant from SD media (pH 4.5) were streaked onto YP plate containing various carbon sources and incubated in air for 4 days. On solid YP plate without carbon sources, half of the white cells transitioned to rod form, whereas YP plate containing glycolytic carbon sources such as glucose, maltose, galactose, or fructose suppressed rod cell formation ([Figure 1B](#)). Interestingly, despite being a glycolytic carbon source, GlcNAc strongly promoted the development of the rod form ([Figure 1B](#)). Among the nonglycolytic carbon sources, ethanol was the most efficient carbon promoting rod formation, whereas citrate, glycerol, and succinate had no impact on rod formation ([Figure 1B](#)). On the YPD plate (YP + glucose), the *efg1* mutant cells were maintained in the gray state, with no discernable rod cell development. However, on YPE plate (YP + ethanol) and YPG plate (YP + GlcNAc), almost all of the *efg1* mutant cells transitioned to the rod phenotype ([Figure 1C](#)). Ethanol appeared to be a robust inducer of rod cell formation, as the *efg1* mutant cells switched to the rod phenotype even at a low concentration of ethanol (0.1%, v/v) and maintained as rod cells through many generations on ethanol-containing plates. As the white, opaque, gray, and rod states represent four distinct morphological pheno-

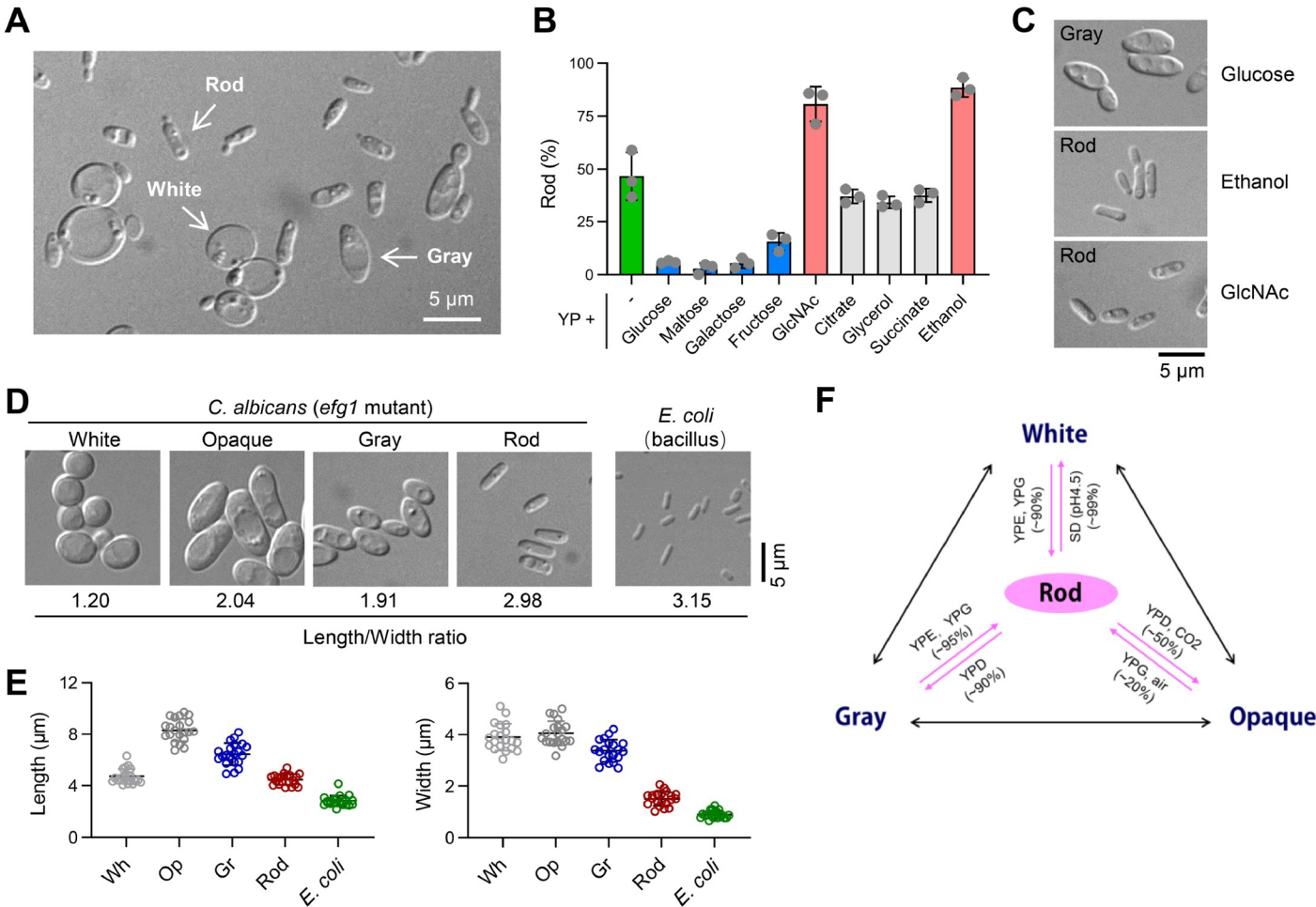


Figure 1. Rod phenotype and the reversible phenotypic switching between rods and white, gray, and opaque in the *efg1* mutant of *C. albicans* (A) Cell morphology of the *efg1* mutant strain (CXY4, *MTLa/a*) plated on solid YP plates and incubated at 25°C for 4 days. Scale bar: 5 μ m. (B) The *efg1* mutant strain (CXY4, *MTLa/a*) was plated on YP media supplemented with various carbon sources (pH 6.8) and incubated at 25°C for 4 days. Cell morphologies were observed under a microscope, and the number of rod-shaped cells was quantified to calculate the rod ratio. (C) Presentation of cell morphologies on YPD (YP + glucose), YPE (YP + ethanol) and YPG (YP + GlcNAc) plates, as illustrated in (B). Scale bar: 5 μ m. (D) Cultivation of *efg1* mutants displaying different morphologies under various conditions for 3 days. White wells were cultured on SD plates (pH 4.5) at 37°C, opaque cells were cultured on YPD plates at 25°C with 20% CO₂, gray cells were cultured on YPD plates at 25°C, and rod cells were cultured on YPE plates at 25°C. *E. coli* was cultured on LB plates at 37°C for 3 days. The ratio of the length to width for white, opaque, gray and rod cells is presented below. Scale bar: 5 μ m. (E) Measurement of the length and width of the white, opaque, gray and rod cells depicted in (D). (F) Diagram illustrating the white-opaque-gray-rod tetra-state switching system under various conditions in *C. albicans*. Different conditions and the switching percentages of different morphologies are indicated in the graph.

types, we then thoroughly assessed their shape and size. As shown in Figure 1D,E, white cells are spherical or ovoid with a length of $\sim 4.7 \mu\text{m}$ and a width of $\sim 3.9 \mu\text{m}$, whereas opaque, gray and rod cells all exhibit a rod-shaped morphology. Among the three rod-shaped cells, opaque cells are the largest (length-width, $8.3 \mu\text{m}$ - $4.1 \mu\text{m}$), and gray cells are moderate in size (length-width, $6.4 \mu\text{m}$ - $3.4 \mu\text{m}$), whereas the rod cells are the smallest, with a length of $4.5 \mu\text{m}$ and a width of $1.5 \mu\text{m}$, resembling the morphology of *Escherichia coli* with a length of $2.8 \mu\text{m}$ and a width of $0.9 \mu\text{m}$. Gray cells had a length/width ratio (L/W, 1.91) similar to that of opaque cells (L/W, 2.04) and greater than that of white cells (L/W, 1.20) but lower than that of rod cells (L/W, 2.98). Interestingly, the length/width ratio of rod cells was similar to that of *E. coli* cells (L/W, 3.15). These measurements provided valuable insights into the characteristics of each phenotype, potentially correlating with their

adaptability in host niches.

Dynamic transitions between rod and white, gray, or opaque were observed under different media and growth conditions. Specifically, white cells were predominant in SD media (pH 4.5) in air, gray cells were the major cell types in YPD media (pH 6.8) in air, opaque cells were stably maintained in YPD or YPG media in air or a high concentration of CO₂, and rod-state cells were prominent in YPE or YPG media in air (Figure 1F). Rod represented a stable phenotype but could also switch to the other three phenotypes reversibly. White and gray switched to rod by more than 90% on YPE or YPG plate at 25°C. Opaque was switched to rod at 20% on YPG plate. Conversely, rod switched to white on SD, to gray on YPD, and to opaque in high CO₂ (Figure 1F). The clinical isolate BJ1097 harbored a mutation (C181T) in the *EFG1* gene, leading to a premature stop codon (Q61*) [15]. Like *efg1* mutant, BJ1097 strain

also exhibited morphological switching between gray, rod, opaque and white in response to different growth conditions (Supplementary Figure S1). Thus, *efg1* deficiency appears to be crucial for the formation of rod cells in both laboratory and clinical strains. These observations collectively indicated that the rod state represents a novel cell morphological phenotype of *C. albicans* that can be stably maintained throughout many generations of cell division. The rod phenotype, in conjunction with the white, gray and opaque phenotypes, is proposed to constitute a tetrastable switching system in *C. albicans*.

Maintenance of the rod phenotype of *efg1* mutant *in vitro* and *in vivo*

To further assess the stability of the rod form, we streaked the rod cells of the *efg1* mutant onto media containing various carbon sources and analyzed the ratio of rod cells. The rod form remained stable on YP, YPE, and YPG plates in air for a 4-day duration. However, the rod cells rapidly converted to the gray form just one day after incubation on YPD plate, with the percentages of gray cells kept increasing over time (Figure 2A). Next, we examined whether rod cells can colonize the gut in mice. Rod cells of the *efg1* mutant were first selected from YPE plates and orally inoculated into antibiotic-pretreated mice. To prevent phenotype switching on different carbon sources, fresh fecal pellets were collected, homogenized and plated on YP plates immediately. The phenotype of the inoculated fungal strain was assessed following incubation in air for one day (Figure 2B). Strikingly, we found that the rod phenotype of the *efg1* mutant was maintained for up to 20 days after inoculation (Figure 2C). Hence, the rod form of the *efg1* mutant can colonize the gut and keep self-renewal as the rod form for an extended period *in vivo*.

Rod cells exhibit a specific gene expression program

To delineate their gene expression profiles, we performed RNA sequencing (RNA-seq) analysis on rod cells and gray cells. Rod cells of the *efg1* mutant were collected from YPE plates, while gray cells of the *efg1* mutant were obtained from YPD plates. We detected a comparable number of transcribed genes in both rod ($n = 5740$) and

gray ($n = 5806$) cells (Figure 3A and Supplementary Table S4). In contrast, comparative analysis revealed more DEGs in rod cells than in gray cells. Specifically, 610 genes were upregulated, while 963 genes were downregulated in rod cells compared with gray cells (Figure 3B and Supplementary Table S5). Compared to gray cells, rod cells exhibited predominant enrichment in cell cycle-related gene expression (Figure 3C). The expression levels of *CDC28* and G1 or G2 cyclins were upregulated in rod cells (Supplementary Figure S2). Additionally, genes involved in the DNA damage response, microtubule binding, and filamentous growth were also enriched in rod cells. Conversely, carbohydrate transport genes were predominantly enriched in gray cells (Figure 3C). Furthermore, we identified a subset of genes specifically expressed in rod cells, such as *CSA2* and *CWP419* (Figure 3E). To validate the rod-specific genes, we conducted RT-qPCR analysis to examine the expression levels of these marker genes in four phenotypic-state cells. As illustrated in Figure 3F, the white-specific gene *WH11*, which was highly expressed in both white and gray cells was strongly downregulated in rod cells. Opaque-specific genes *WOR1* and *OP4* were also downregulated in rod cells. Conversely, *CSA2*, *CWP419*, *PGA26* and *LIP9* were highly expressed in rod cells. Notably, *HSP31*, *LIP9*, and *PGA26* were previously considered gray-specific markers [5]. This observation could be attributed to the presence of a mixed population of rod and gray cells in the previous report. Thus, our data provide a comprehensive understanding of the transcriptomic features associated with the rod phenotype of *C. albicans*. More importantly, the identification of specific genes related to rod cell formation and potential marker genes provides insights into the molecular mechanisms underlying this unique morphological form of *C. albicans*.

GlcNAc sensor Ngs1 is required for rod cell formation of *efg1* mutant in YPG

GlcNAc serves as a major structural component on the cell surface of a wide range of cells, including the bacterial cell wall and animal cell-secreted extracellular matrix [30]. GlcNAc is proposed to be a primary carbon source supporting the growth of *C. albicans* in humans [31]. In addition to its role as an energy source, GlcNAc

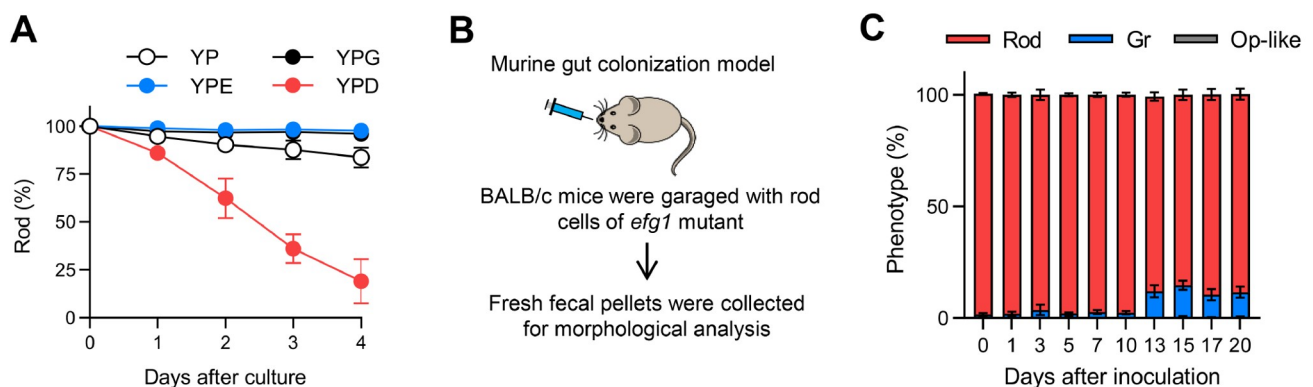


Figure 2. The rod phenotype of the *efg1* mutant is maintained in growth media and during gut colonization in mice (A) The *efg1* mutant strain (CX4, *MTLa/a*) was cultured at 25°C on YPE (YP + ethanol) plates for 2 days to induce rod morphology. Subsequently, the rod-shaped cells were streaked on YP, YPD, YPE, and YPG plates to observe the cell morphology over 1 to 4 days. (B) The *efg1* mutant (CX4, *MTLa/a*) cells were cultured on YPE plates for 2 days to induce rod morphology. Subsequently, 1×10^8 rod cells were orally administered to 6- to 8-week-old BALB/c mice ($n = 3$ to 5 mice per group) treated with antibiotics. Fresh fecal pellets were collected and plated on YP agar plates supplemented with antibiotics at 25°C for one day. The strain morphologies (phenotypes) were tracked over a 20-day period. (C) The ratio of different morphologies was quantified and calculated.

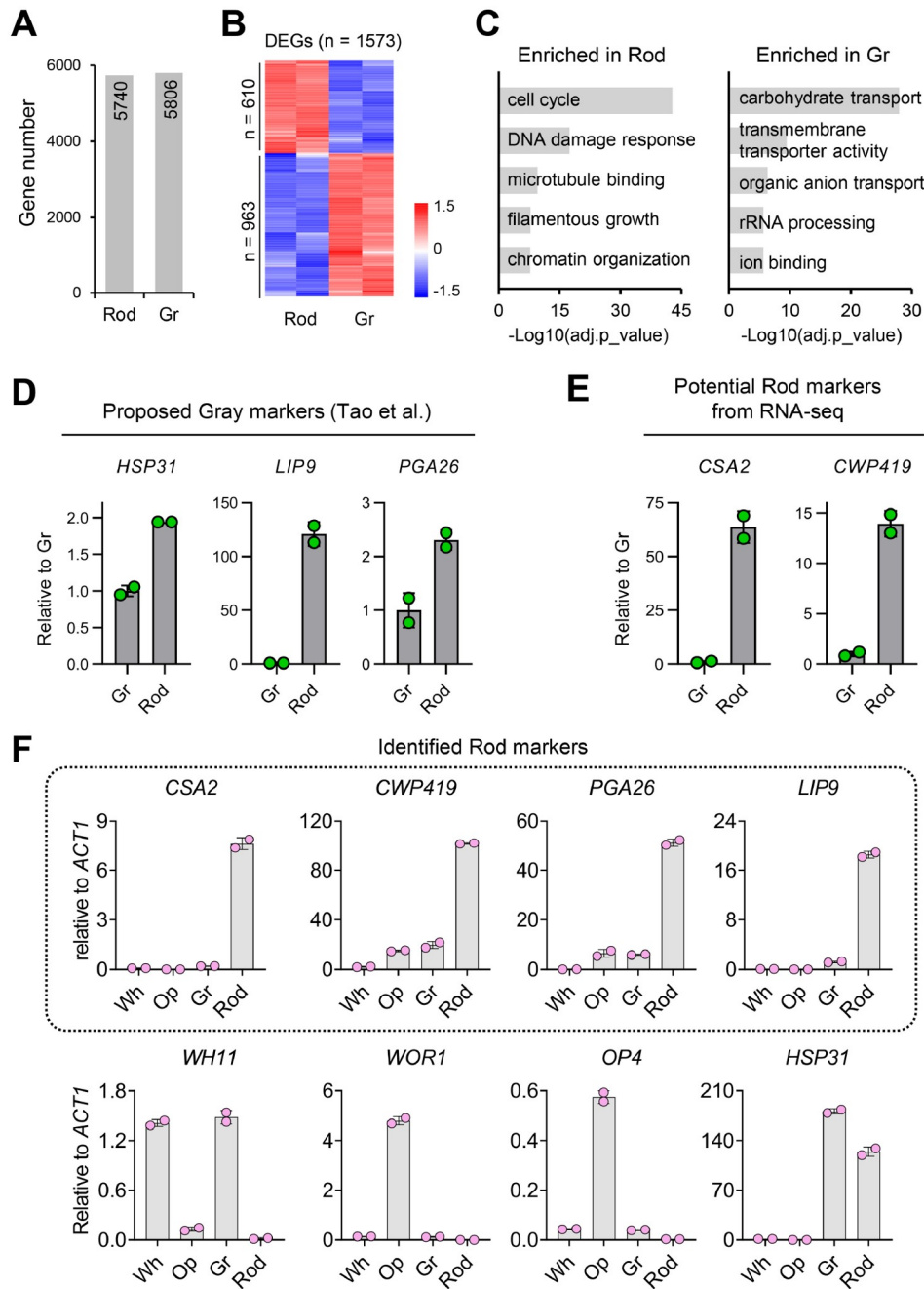


Figure 3. Comparison of rod and gray cell gene expression (A-E) The *efg1* mutant strain (CXY4, *MTLa/a*) was plated onto YPD and YPE plates and incubated at 25°C for 3 days in air to obtain rod and gray cells. Subsequently, the cells were collected for RNA sequencing (RNA-seq) analysis. Differentially expressed genes were identified using DESeq2 (version 1.34.0 in RStudio) with criteria of a fold change ≥ 2 and a *P* value of < 0.05 . (F) Various morphologies of the *efg1* mutant strain (CXY4, *MTLa/a*) were cultured under different conditions at 25°C for 2 days. Rod cells were cultured on YPE plates, and gray cells were cultured on YPD plates. Subsequently, the cells were collected for qRT-PCR analysis.

can act as a potent inducer of morphological transitions in *C. albicans*, including hyphal development [32] and phenotypic switching [21]. Ngs1, the GlcNAc sensor and transducer, governs GlcNAc-induced expression of genes involved in GlcNAc catabolism, uptake, scavenging, and pathogenesis [30]. To examine the functional role of Ngs1 in rod formation, we deleted the *NGS1* gene in the *C. albicans* *MTLa/a* strain and investigated its cell morphology on GlcNAc-containing media. The *ngs1* mutant cells

exhibited white phenotype on YPG or YPE plates when grown in air condition (Figure 4A). Since the *efg1* mutant can form rod cells on YPG or YPE plate, we subsequently deleted the *NGS1* gene in the *efg1* mutant to construct the *efg1/ngs1* double mutant for further analysis. Interestingly, the deletion of *NGS1* in the *efg1* mutant inhibited the formation of rod cells on YPG but not on YPE or YP plate (Figure 4A,B). The *efg1/ngs1* double mutant cells mainly exhibited a gray phenotype in YPG media. However, the main

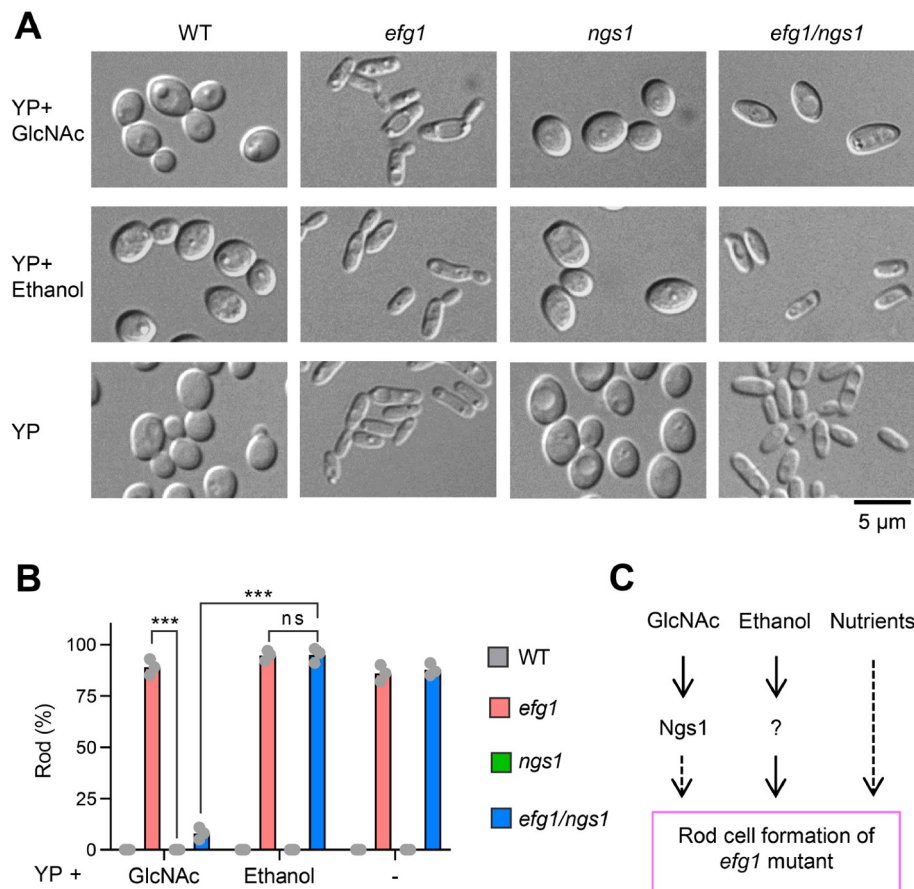


Figure 4. Deletion of *NGS1* in the *efg1* mutant inhibits rod cell formation in the YPG (A) Wild-type (JYC5 + vector, *MTLa/a*), *efg1* (CXY4, *MTLa/a*), *ngs1* (CXY36, *MTLa/a*), and *efg1/ngs1* (CXY30, *MTLa/a*) mutants were plated onto YPD and YPE plates and incubated at 25°C in air for 2 days to observe cell morphology. Scale bar: 5 μ m. (B) The cells in (A) were collected to calculate the ratio of rod cells. (C) GlcNAc facilitates the formation of rod cells through the GlcNAc sensor Ngs1, while ethanol and nutrients may augment rod cell formation *via* alternative pathways.

population of *efg1/ngs1* double mutant cells still switched to the rod form on YPE or YP plates. These results suggested that Ngs1 plays an essential role in promoting GlcNAc-induced rod formation in the *efg1* mutant but is dispensable for ethanol- or other nutrient-induced rod formation (Figure 4C). Therefore, in addition to its well-established roles in yeast-hyphae transition and white-opaque switching, Ngs1 is also required for inducing the rod form in response to GlcNAc.

The transcription factor Cph1 mediates the repression of glucose on rod form induction of *efg1* mutant

The above data demonstrated that Efg1 is a master suppressor of rod cell formation. During filament formation, deletion of *CPH1* in the *efg1* mutant resulted in a failure to form filaments under all tested conditions [7]. To explore whether Cph1 is also required for rod cell formation in the *efg1* mutant, we examined the phenotype of *efg1/cph1* mutant (*MTLa/a*) cells in various YP medium. We observed that the *efg1/cph1* mutant transitioned to rod form and maintained this phenotype in air at 25°C. The *efg1/cph1* mutant maintained rod phenotype not only on YP, YPE, or YPG plate but also on YPD plate (Figure 5A,B and Supplementary Figure S4), suggesting that the loss of Cph1 in the *efg1* mutant relieved the inhibitory effect of glucose on rod formation. Importantly, the deletion of *CPH1* alone in wild-type

cells had no obvious effect on rod form induction (Figure 5A,B). These findings indicate that Efg1 functions as a broader suppressor of rod cell formation at higher levels, whereas Cph1 is primarily involved in glucose-mediated suppression of rod cell formation (Figure 5C).

The rod cells of the *efg1/cph1* mutant can colonize and self-renew in the gut microenvironment

The rod cells of the *efg1/cph1* mutant exhibited a stable rod phenotype on YPD plate or other YP plates for numerous generations at 25°C in air. However, at physiological temperature (37°C), the rod form of *efg1/cph1* mutant cells tended to transition into larger forms, including white, gray and opaque (Supplementary Figure S4). Particularly in YPG plate with a high concentration of CO₂, the rod form of the *efg1/cph1* mutant switched to gray and opaque-like forms at 37°C. To analyze the phenotype of the *efg1/cph1* mutant (*MTLa/a*) at physiological temperature (37°C) *in vivo*, we generated a mouse gut colonization model, as described in Figure 2B. The *efg1/cph1* cells from both the YP and YPD plates maintained the original rod phenotype for up to 20 days of gut colonization (Figure 6). This observation demonstrated the adaptability of *efg1/cph1* rod cells to the host gut microenvironment.

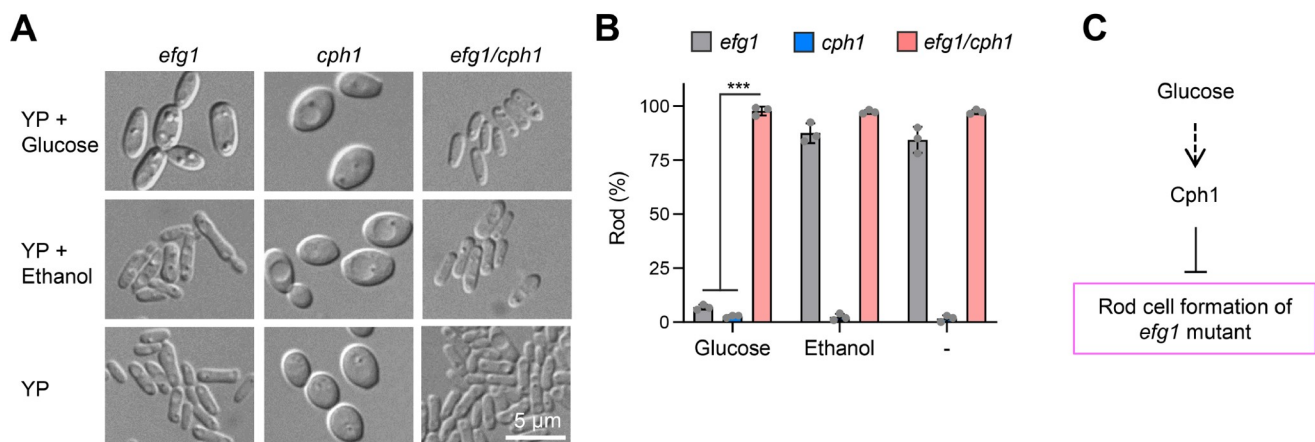


Figure 5. Deletion of *CPH1* in the *efg1* mutant induces rod cell formation in YPD (A) The *efg1* (CXY4, *MTLa/a*), *cph1* (CXY35, *MTLa/a*) and *efg1/cph1* (CXY33, *MTLa/a*) mutants were plated onto YPD and YPE plates and incubated at 25°C in air for 2 days to observe cell morphology. Scale bar: 5 μ m. (B) The cells in (A) were collected to calculate the ratio of rod cells. (C) Schematic representation of *CPH1* inhibition of rod cell formation in the *efg1* mutant on glucose-containing plates.

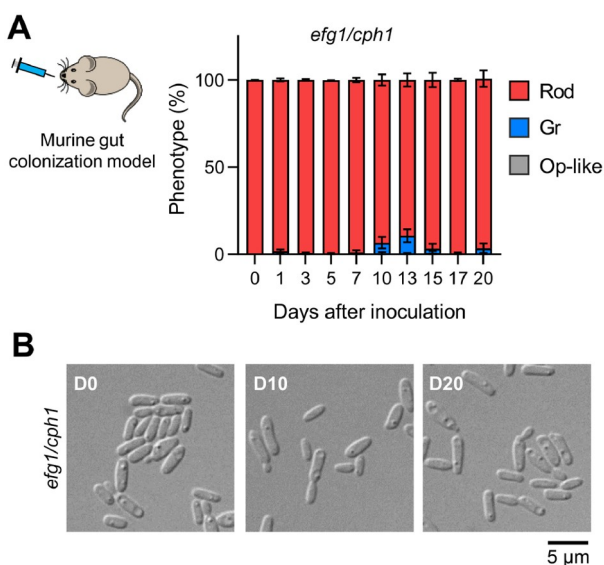


Figure 6. Colonization and morphology of *efg1/cph1* rod cells in the mouse gut (A) Schematic representation of the commensalism experiments shown on the left. The *efg1/cph1* (CXY33, *MTLa/a*) mutant cells were cultured on YPD plates for 2 days to induce rod morphology. Subsequently, 1×10^8 rod cells were orally administered to 6- to 8-week-old BALB/c mice ($n = 3$ to 5 mice per group) treated with antibiotics. Fresh fecal pellets were collected and plated on YP agar plates supplemented with antibiotics at 25°C for 1 day. The strain morphologies (phenotypes) were tracked over a 20-day period. (B) Representative cell morphologies are displayed.

The rod cells of *efg1/cph1* mutant showed superior fitness to the host gut than white cells

Previous research has indicated that the adaptability of fungal cells to the gut can be improved by the loss of the functional *EFG1* allele [15–18]. To investigate whether the rod form of the *efg1/cph1* mutant confers enhanced fitness in the gut environment, we performed a competition experiment with the wild-type (WT) strain

in a mouse model (Figure 7A). In this experimental setup, mice were orally inoculated with an equal ratio of wild-type (*MTLa/a*) white cells and *efg1/cph1* mutant (*MTLa/a*) rod cells. Then, fresh fecal pellets were collected daily from the mice, homogenized and plated onto YPD plate. Following a 24-h incubation at 25°C, the competitive index (CI) representing the ratio of the indicated strains was determined by specific primers designed based on specific inserted sequences in the genome (Supplementary Table S3). As expected, the *efg1/cph1* mutant displayed superior fitness to the gut, outcompeting WT cells in the GI tract (Figure 7B). The *efg1/cph1* mutant cells exhibited rapid expansion and pronounced advantages over the wild-type cells starting one day postinoculation in the GI tract. In line with the observed genotype, the cells recovered from the fecal pellets constantly appeared in rod form on YP plate. These data suggested that the rod form of the *efg1/cph1* mutant had a competitive advantage over the white form of the WT in the GI tract.

Discussion

In this study, we revealed a novel “rod” form in *C. albicans* that morphologically resembles opaque and gray forms but is the smallest in size. The rod phenotype manifests a specific gene expression program, superior adaptability to the GI niche and long-lasting genetic stability, thus constituting a tetra-stable switching system along with white, gray, and opaque phenotypes (Figure 1F). Despite sharing a rod-shaped morphology with opaque and gray cells, rod cells are much smaller and reminiscent of the bacillus morphology observed in bacteria.

The mammalian gut presents a heterogeneous environment, with various nutrients being longitudinally absorbed in various anatomic locations. Notably, glucose becomes relatively scarce in the colon, where gut bacteria are enriched and can shed large amounts of the cell-wall component GlcNAc. Coincidentally, we found that the transition of the *efg1* mutant of the SC5314 strain and the more recently isolated clinical strain BJ1097 to a smaller rod form is contingent on GlcNAc-sufficiency and glucose deficiency, reminiscent of the colonic microenvironment. The identification of Ngs1 as a crucial component connecting GlcNAc signaling to the rod form

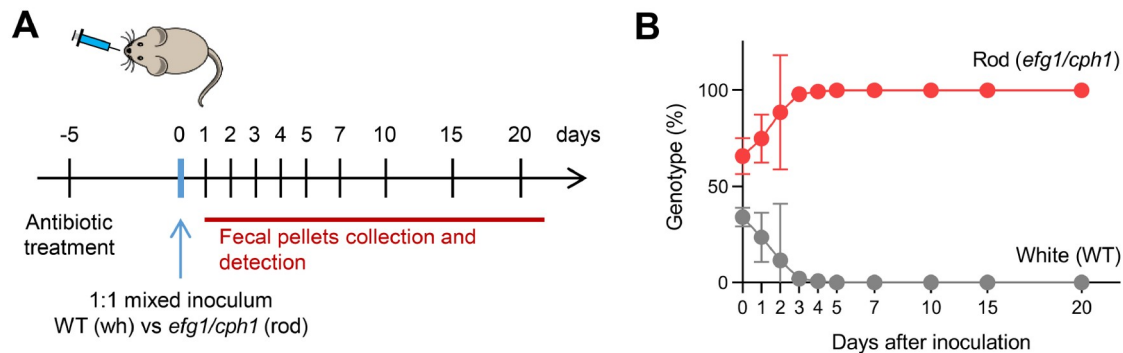


Figure 7. Rod cells exhibit a fitness advantage in a mouse competitive commensal GI model (A) Schematic representation of the competitive fitness experiments. Wild-type (JYC5 + Vector, *MTLa/a*) and *efg1/cph1* (CX33, *MTLa/a*) cells were cultured on YPD plates for 2 days. Subsequently, 1×10^8 cells were orally administered with 1:1 mixtures of wild-type (JYC5 + Vector, *MTLa/a*) and *efg1/cph1* (CX33, *MTLa/a*) cells to 6- to 8-week-old BALB/c mice ($n=3$ to 5 mice per group) that were treated with antibiotics. Fresh fecal pellets were collected and plated on YP agar plates supplemented with antibiotics at 25°C for 1 day. (B) The relative abundance (genotype) of the strains was tracked over a 20-day period.

adds another layer of complexity to the comprehensive molecular mechanisms governing phenotypic switching in *C. albicans*. The deletion of *CPH1* in the *efg1* mutant also revealed the involvement of the mating MAP kinase pathway in modulating rod formation in response to glucose depletion.

In line with its carbon source preference, the rod form of *C. albicans* demonstrated superior commensalism and competitive fitness by outcompeting white cells during gut colonization in our study. This observation aligns with previous reports suggesting a competitive fitness advantage of gut-evolved nonfilamentous *C. albicans*. Previous reports suggested a competitive fitness advantage and cross-protective innate immunity of gut-evolved nonfilamentous *C. albicans* [22,23]. Building on this evidence, we tentatively posit that this naturally avirulent rod form, with its advantage in colonization and propagation in the host gut, may enhance the production of host immune factors central to commensalism, providing the host with better protection against pathogenic infections.

The mammalian intestine harbors trillions of microorganisms, including bacteria and fungi, which have coevolved with the host in a symbiotic relationship. In adults, the major phyla among intestinal eubacteria include Gram-negative *Pseudomonadota*, *Bacteroidota*, and Gram-positive *Bacillota* [33]. Although fungi constitute a small proportion of the overall microbial community in the gastrointestinal ecosystem, they exert significant effects on gut metabolite levels, influencing host physiology and the immune system. *C. albicans* is believed to establish long-term colonization in the human gut microbiome without causing disease [34]. Given that fungal cells are typically larger than bacterial cells, switching to a smaller rod form may confer adaptive advantages for *C. albicans* in the mammalian gut environment. This morphological shift could conceivably be beneficial to competition for limited space and nutrients in the gut, thus facilitating fungal cosurvival with bacteria.

Cell size is under precise control and has been linked to cell cycle progression and growth. In the budding yeast *Saccharomyces cerevisiae*, size control primarily occurs at the G1-S transition [35,36]. The G1 cyclin Cln3 acts as a sizer, coordinating the start of the cell cycle with cell growth and nutrient availability. Cln3 induces the expression of *CLN1* and *CLN2* through a transcription activator complex. The burst of *CLN1* and *CLN2* transcription is

delayed in rich media, leading to a larger size at the start of the cell cycle. The addition of glucose or cAMP represses the transcription of *CLN1*, effectively delaying its initiation. Thus, Cln1 and Cln2 play specific roles in integrating environmental signals within the G1-S module. In the fission yeast *Schizosaccharomyces pombe*, size control predominantly involves the G2-M transition [35,36]. As cells traverse through the S and G2 phases, the B-type cyclin Cdc13 gradually accumulates. However, CDK1 activity is inhibited by Wee1-dependent tyrosine phosphorylation. The mitotic inhibitory kinase Pom1 hinders the pro-mitotic kinase Cdr2, and Cdr2, in turn, inhibits the Wee1 kinase. As *S. pombe* cells elongate, the decreasing medial concentration of Pom1 enables the activation of Cdc13-CDK1, facilitating entry into mitosis. While rod-shaped *C. albicans* cells generate daughters through budding, akin to the budding yeast *S. cerevisiae*, their morphology is reminiscent of that of the rod-shaped fission yeast *S. pombe*. Consequently, the cell size control mechanisms in *C. albicans* rod-shaped cells may be regulated at both the G1-S transition and the G2-M transition. Indeed, RNA-seq analysis revealed that rod cells exhibited greater cell cycle-related gene expression than gray cells. The upregulation of *CDK1* (Cdc28) and G1 cyclins (*CLN1* and *CLN2*), as well as G2 cyclins (*CLB2* and *CLB4*), in rod cells (Supplementary Figure S2) reflects the influence of cell cycle regulation on the cell size control of *C. albicans* rod-shaped cells. Recent reports have highlighted that yeast cell size homeostasis is regulated across multiple layers, including cell cycle progression, protein synthesis, RNA transcription, and mRNA stability [37,38]. Consequently, various environmental signals impinge on cell size control, which could be a selective trait crucial for survival in fluctuating, nutrient-limited environments. Nevertheless, the potential link between the altered cell cycle of rod cells and their morphology warrants further study.

In addition to differences in cell cycle regulation between rod and gray cells, *LIP9* was identified as a distinctive marker exclusive to rod cells among all four cell types. Extracellular lipolytic activity provides *C. albicans* with the ability to utilize lipids as the sole source of carbon. The *C. albicans* genome harbors a substantial gene family comprising ten lipase genes [39,40]. No homologous genes were found in the nonlipolytic yeast *S. cerevisiae*. However, when the *C. albicans* *LIP1* gene was introduced into *S. cerevisiae*, *S. cerevisiae* was able to produce a secreted lipase that hydrolyzed

Tween and triacylglycerols [39]. Therefore, large *LIP* gene family members might have evolved to adapt to the permanent association of *C. albicans* with the human host. Among the ten *LIP* genes (Supplementary Figure S3), *LIP9* was highly induced in the rod cells of *C. albicans*, suggesting that rod cells may be able to convert dietary fats to free fatty acids for absorption in the small intestine, thereby contributing to host health.

In conclusion, this study established the rod form as a novel and stable morphotype of *C. albicans*, contributing to its adaptability in the host gut. The identified molecular markers and genetic/environmental determinants associated with the rod form lay a foundation for future explorations of the far-reaching implications of these findings in the context of microbial behavior, host-microbe interactions, and therapeutic interventions.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (Nos. 32170196 and 32030040), the National Key Research and Development Program of China (No. 2021YFA1301400), and the Shanghai Municipal Science and Technology Major Project (No. 2019SHZDZX02).

Conflict of Interest

The authors declare that they have no conflict of interest.

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