Iron-depletion promotes mitophagy to maintain mitochondrial integrity in pathogenic yeast *Candida glabrata*

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ABSTRACT
*Candida glabrata*, a haploid budding yeast, is the cause of severe systemic infections in immunocompromised hosts. The amount of free iron supplied to *C. glabrata* cells during systemic infections is severely limited by iron-chelating proteins such as transferrin. Thus, the iron-deficiency response in *C. glabrata* cells is thought to play important roles in their survival inside the host’s body. In this study, we found that mitophagy was induced under iron-depleted conditions, and that the disruption of a gene homologous to *ATG32*, which is responsible for mitophagy in *Saccharomyces cerevisiae*, blocked mitophagy in *C. glabrata*. The mitophagic activity in *C. glabrata* cells was not detected on short-period exposure to nitrogen-starved conditions, which is a mitophagy-inducing condition used in *S. cerevisiae*. The mitophagy-deficient *atg32Δ* mutant of *C. glabrata* also exhibited decreased longevity under iron-deficient conditions. The mitochondrial membrane potential in *C. glabrata* cells was significantly lower than that in wild-type cells under iron-depleted conditions. In a mouse model of disseminated infection, the *Cgatg32*Δ strain resulted in significantly decreased kidney and spleen fungal burdens compared with the wild-type strain. These results indicate that mitophagy in *C. glabrata* occurs in an iron-poor host tissue environment, and it may contribute to the longevity of cells, mitochondrial quality control, and pathogenesis.

Introduction
The number of immune-compromised individuals has increased because of HIV infection and population aging, and this clinical situation has provided opportunities for systemic infections by *Candida* spp. In candidiasis, a new emerging trend has been observed, where there has been a shift toward infections with non-*albicans* *Candida* species. In particular, *C. glabrata* is now the second most important cause of fungal infections in humans. However, little is known about the virulence factors that contribute to the pathogenesis of *C. glabrata*.

Mitochondria play a central role in cellular energy metabolism, where these organelles are essential for the generation of the ATP required for multiple cellular functions. They consume large amounts of molecular oxygen, and the mitochondrial respiratory chain is the major source of cytotoxic reactive oxygen species (ROS). Thus, controlling the quality and quantity of mitochondria is essential in every eukaryotic organism.

Autophagy can be classified as bulk or selective. Mitophagy, the degradation of mitochondria via selective autophagic machinery, is thought to be involved in regulating the mass and function of mitochondria. Mitophagy in yeast cells is efficiently activated under nitrogen-starved conditions (following growth on a nonfermentable carbon source) or after long-term growth in nonfermentable medium conditions. However, the physiological importance of mitophagy in this model organism and other yeast species remains largely unexplored. The mitochondrial protein Atg32, which is responsible for mitophagy, was identified in *S. cerevisiae*, and studies of Atg32 have helped to elucidate the detailed processes of mitophagy.

Mitophagy is required for the longevity of *S. cerevisiae* during caloric restriction. The mechanisms that confer longevity by mitophagy are considered to be related to the elimination of dysfunctional mitochondria. The life span of yeast cells is determined by the cellular ROS and glutathione (GSH) levels, mitochondrial membrane potential (MMP), and the concentration of glucose in the medium, but the details of the longevity-promoting mechanisms have not been fully elucidated.

In this study, we showed that the expression of *C. glabrata* (*Cg*) *ATG32* was upregulated and mitophagy was activated under iron-deficient conditions in *C. glabrata*. We compared the mitophagic activity, ROS production, MMP, and chronological life span (CLS)
of wild-type (WT) and atg32Δ mutant isogenic C. glabrata strains under iron-depleted conditions. Finally, we examined the importance of mitophagy in C. glabrata using mouse infection models.

Results

**Both the expression and phosphorylation of CgAtg32 are increased under iron-depleted conditions in Candida glabrata**

During systemic infections, the amount of free iron supplied to C. glabrata cells is thought to be severely limited by iron-chelating proteins such as transferrin. Thus, the iron-deficiency response in C. glabrata cells and other pathogenic microorganisms probably has important roles in their survival inside the host’s body. To investigate the response to iron deficiency in C. glabrata, we performed transcriptome analyses of cells grown under iron-replete (synthetic glucose medium; SD) and iron-depleted (SD medium without iron but with 100 μM ferrozine, which is a chelator of iron; SD-Fe) conditions. The expression level of a gene orthologous to ATG32, the essential gene for mitochondria-specific autophagy (mitophagy) in Saccharomyces cerevisiae, CAGL0H06545g/CgATG32, was upregulated in C. glabrata cells grown in SD-Fe medium (Table S1).

To verify the results of the transcriptome analysis, we investigated the expression of CgATG32 under iron-depleted conditions by real-time RT-PCR. The expression level of CgATG32 was 3-fold greater in cells grown in SD-Fe medium for 4 h compared with cells grown in SD medium (Fig. 1A). We further examined the expression level of CgATG32 and CgATG8 both of which are indispensable proteins for mitophagy during longer incubation periods (Fig. S2A, B). Time-course experiments showed that the amount of RNA of both CgATG32 and CgATG8 were decreased at 8-h incubation with SD-Fe, and increased again after a 24-h incubation.

It has been reported that the factors for mitophagy-induction in yeast are not only the expression level of ATG32 but also the phosphorylation status of the corresponding protein. Next, we performed western blot analysis to examine the phosphorylation status of CgAtg32 using a hemagglutinin (HA)-tagged CgATG32 expressing strain (atg32Δ 3HA-CgATG32) (Fig. 1B). The apparent molecular mass of CgAtg32 on a SDS polyacrylamide gel was increased when the cells were grown either in SD or in SD-Fe medium on d 1. CgAtg32 was not detected after 2 d of incubation in SD-Fe medium whereas it was gradually decreased but still detectable on d 7 in SD medium. To determine whether these electrophoretic mobility shifts of CgAtg32 were due to phosphorylation, cellular protein extracts were treated with lambda protein phosphatase (λ PP) (Fig. 1C). The shifted CgAtg32 bands observed on day 0 and on d 1 with the test medium (SD or SD-Fe) disappeared following λ PP treatment, indicating that the shifted-band reflected the phosphorylated CgAtg32. Furthermore, the amount of both total and phosphorylated CgAtg32 was much higher in SD-Fe conditions than in iron-replete conditions. These results suggest that CgAtg32 is phosphorylated even in SD medium, and iron deficiency increases the amount of CgAtg32 and promotes further phosphorylation of this protein. Thus, we hypothesized that mitophagy was induced in C. glabrata under iron-depleted conditions and that mitochondrial degradation may play roles in the adaptation to iron deficiency.

**Mitophagy is induced under iron-depleted conditions**

We examined mitophagy in C. glabrata cells under iron-depleted conditions. Cells expressing mitochondria-targeted mouse dihydrofolate reductase (AccΔ; V00734.1)-GFP (mtDHFR-GFP) were grown in SD or SD-Fe medium, and proteolytic processing of mtDHFR-GFP was detected by western blotting with anti-GFP antibody. It is considered that mtDHFR-GFP is degraded to produce the GFP moiety when mitophagy (involving the vacuolar degradation of mitochondria) is induced. The processed GFP was detected in cells grown in SD-Fe medium for 36 and 48 h, but not in SD medium (Fig. 1D). We also performed a mtDHFR-GFP processing assay with mutant cells that lacked the orthologous genes of S. cerevisiae ATG32 (Cgatg32Δ), ATG1 (Cgatg1Δ), and ATG11 (Cgatg11Δ). In S. cerevisiae, ATG1 is indispensable for bulk and selective autophagy, whereas Atg11 is required primarily for the latter. The release of GFP from mtDHFR-GFP in SD-Fe medium at 48 h was eliminated completely in every atg null mutant (Fig. 1E). Furthermore, we validated the GFP localization to the vacuole in WT cells but not in Cgatg32Δ cells grown with SD-Fe by fluorescence microscopy observation (Fig. S3A). These results suggest that mitophagy is induced in C. glabrata under iron-depleted conditions, and that CgATG32, CgATG1, and CgATG11 are essential genes for mitophagy in C. glabrata as well as in S. cerevisiae.

**Mitophagy is induced in long-term culture, but is not immediately induced by nitrogen starvation**

In S. cerevisiae, mitophagy is immediately induced (2–4 h) when cells are cultured under nitrogen-starved conditions after preculturing in nonfermentable medium. In addition, it has also been reported that nonfermenting long-term culture (>2 d) induce mitophagy. We performed the mtDHFR-GFP processing assay with C. glabrata cells grown in long-term culture or in nitrogen-starvation medium. The released GFP moiety was detected using cells grown in nonfermentable medium with glycerol (YPG) for 2–5 d (Fig. 2A) as well as in S. cerevisiae. The processing of mtDHFR-GFP was not detected in Cgatg32Δ mutant cells grown in YPG medium (Fig. 2A), indicating that CgATG32 is also responsible for mitophagy in nonfermented long-term culture. Conversely, the released GFP was not detected using cells grown in nitrogen-starvation medium (SD-N) for 0–6 h (Fig. 2B). For longer incubation (1–3 d) in SD-N medium, the free GFP was finally detected in C. glabrata (Fig. S4B; longer exposure image of blot). The mtDHFR processing assay also showed that the amount of processed GFP moiety under iron-depleted conditions was substantially higher than that observed in nonfermentative or nitrogen-starved long-term culture (Fig. S4A, C). These results suggest that iron depletion rather than long-term culture or nitrogen-starved conditions strongly induces mitophagy in C. glabrata. In contrast, iron depletion did not promote mtDHFR-GFP processing in S. cerevisiae (Fig. S4D). These data suggest that the extracellular signals that potentially induce mitophagy are partly conserved yet different between S. cerevisiae and C. glabrata.
Inhibition of mitochondrial functions does not induce mitophagy

The respiratory activity of \textit{C. glabrata} cells grown in SD-Fe medium is thought to be decreased because some key respiratory enzymes require iron for their function.\textsuperscript{25,26} Thus, we hypothesized that mitochondrial degradation in \textit{C. glabrata} is also induced by respiratory inhibition. However, no mtDHFR-GFP processing was observed in anaerobically cultured cells (Fig. 3). Subsequently, the mtDHFR-GFP processing assay was performed using cells grown under conditions where mitochondrial function was attenuated. The addition of carbonyl cyanide \textit{m}-chlorophenyl hydrazone, an uncoupler of oxidative phosphorylation, or antimycin A, a mitochondrial electron transport inhibitor, did not induce mitophagy (Fig. 3). These results indicate that mitochondrial dysfunction alone could not initiate mitophagy in \textit{C. glabrata}.

\textit{CgAtg32} is required for longevity under iron-depleted conditions

It has been reported that mitophagy confers longevity on \textit{S. cerevisiae} under nitrogen-starved conditions.\textsuperscript{11} Thus, we
The amount of cellular ROS increases in the conditions. Thus, we hypothesized that mitophagy decreases cellular ROS accumulation in medium with or without the indicated supplements (4 \( \mu \)g/mL carbonyl cyanide m-chlorophenyl hydrazone [CCCP]; 8 \( \mu \)g/mL antimycin A), or under anaerobic growth condition, collected at the indicated time points, and then subjected to western blot analysis with anti-GFP and anti-CgPgk1 (loading control) antibodies.

Inhibition of respiration or mitochondrial functions do not induce mitophagy in \textit{C. glabrata}. The MMP was quantified by staining cells with the fluorescent membrane potential-dependent dye 3,3’dihexyloxacarbocyanine iodide (DiOC\(_4\)) and the fluorescence was evaluated using a flow cytometer. In SD medium, both the mean cellular MMP levels and the ratio of MMP-positive cells did not differ significantly between WT, \textit{Cgatg32}\( \Delta \) and \textit{Cgatg32}\( \Delta \) \textit{CgATG32} cells (Figs. 6A, B, S6A, B). Iron-depletion decreased the 2 MMP parameters in the cells of all 3 strains, but the parameters for the WT and \textit{Cgatg32}\( \Delta \) \textit{CgATG32} cells were higher than those for the \textit{Cgatg32}\( \Delta \) mutant under iron-deficient conditions (Figs. 6A, B, S6C, D). These results suggest that mitophagy is required for MMP maintenance under iron-deficient conditions.

**Mitophagy is involved in MMP maintenance under iron-deficient conditions**

The major component of the mitochondrial electrochemical potential gradient of protons is MMP, which is a parameter used to assess mitochondrial function and cellular longevity. The MMP was quantified by staining cells with the fluorescent membrane potential-dependent dye 3,3’dihexyloxacarbocyanine iodide (DiOC\(_4\)) and the fluorescence was evaluated using a flow cytometer. In SD medium, both the mean cellular MMP levels and the ratio of MMP-positive cells did not differ significantly between WT, \textit{Cgatg32}\( \Delta \) and \textit{Cgatg32}\( \Delta \) \textit{CgATG32} cells (Figs. 6A, B, S6A, B). Iron-depletion decreased the 2 MMP parameters in the cells of all 3 strains, but the parameters for the WT and \textit{Cgatg32}\( \Delta \) \textit{CgATG32} cells were higher than those for the \textit{Cgatg32}\( \Delta \) mutant under iron-deficient conditions (Figs. 6A, B, S6C, D). These results suggest that mitophagy is required for MMP maintenance under iron-deficient conditions.

**Disruption of \textit{CgATG32} reduces the virulence of \textit{C. glabrata} in a mouse model of disseminated infection**

The possible role of mitophagy during disseminated infection was tested in a mouse infection model. Mice were inoculated intravenously with the WT strain, \textit{Cgatg32}\( \Delta \) mutant, or...
revertant cells (the Cgatg32Δ mutant transformed with CgATG32). In mice infected with the Cgatg32Δ strain, the number of cells recovered from the kidneys was about 6 times lower than that from mice infected with either the WT or the revertant strain (Fig. 7A). The cell numbers recovered from the spleen when infected with the 3 strains were similar to the results obtained with the kidneys (Fig. 7B).

The expression of CgATG32 was also examined in cells recovered from the kidneys of immune-compromised mice infected with the WT strain. The CgATG32 expression level was about 6 times greater in cells recovered from mouse kidneys compared with that in cells grown in vitro without supplements (Fig. 7C). These results suggest that mitophagy in C. glabrata cells is an indispensable event for survival inside host organs.

**Discussion**

During systemic infections, the amount of free iron supplied for the growth of C. glabrata cells is severely suppressed by iron-chelating proteins such as transferrin.17 It is considered that the iron deficiency response in C. glabrata cells is indispensable for their survival inside the host’s body. Our transcriptome analysis showed that the expression level of the gene homologous to S. cerevisiae ATG32, which encodes a mitochondrial outer membrane protein required for the initiation of mitophagy, was upregulated under iron-depleted conditions in C. glabrata (Fig. 1A, Table S1). This raised the possibility that mitophagy contributes to the stress responses in C. glabrata cells in an iron-deficient environment.

The mtDHFR-GFP processing assay and microscopy observation of the C. glabrata strain expressing mtDHFR-GFP supported the existence of mitophagy (selective vacuolar mitochondria degradation) in C. glabrata grown under iron-depleted conditions (in SD-Fe). To obtain other evidence for mitophagy, a mitochondrial inner membrane protein, CgCox2, and an outer membrane protein, CgPor1, were quantified by western blotting to examine the amount of mitochondria (Fig. S3B). The amount of CgPor1 seemed to be constant even when mitophagy was activated (day 2–7) whereas that of CgCox2 was gradually decreased until d 7. The decrease of CgCox2 was also observed in cells grown under iron-replete conditions (SD medium) at a slower rate than in cells grown in SD-Fe. The slow decrease of CgCox2 in SD medium may reflect the low mitophagic activity (highly activated mitophagy resulted in a fast decrease of CgCox2 in SD-Fe) (Fig. 4B). The uneven change in the amount of inner and outer mitochondrial membrane protein indicates a change in quality (or structure) but not in quantity of mitochondria. These observations demonstrated an interesting aspect of mitophagy, and further characterization of mitochondria should be performed.

We added 100 μM ferrozine to the iron-depleted medium to mimic the free ferric ion concentration inside the host body. The concentration of free ferric ion inside human serum was thought to be extremely low (∼10−24 M).27 A strong iron chelator was
required for almost complete depletion of free ferric ion. However, it seemed to be difficult to obtain perfectly reproducible results from cells grown in SD-Fe probably due to the huge amount of iron supplied from the nutrient-rich preculture prior to shifting to SD-Fe. For example, the GFP moiety in the mtDHFR-GFP processing assay was detected on d 2 after being shifted to SD-Fe in most experiments, whereas it was detected on d 3 in Figure 4B. The amount of GFP-moiety detected on d 2 might be easy to cause to fluctuate by subtle differences in growth conditions, as they were much lower than those on d 4–5. We assume the results obtained from cells grown in SD-Fe were virtually reproducible; however, other iron-depleted conditions should be tested for further examination.

Because the genes related to autophagy in S. cerevisiae are highly conserved in C. glabrata, we expected that autophagy and mitophagy would involve similar molecular mechanisms in both yeast species. Disruption of the genes homologous to S. cerevisiae ATG32, ATG1, and ATG11 (required for mitophagy, all types of autophagy, and selective autophagy, respectively) blocked mitochondrial degradation completely (Fig. 1E). The phosphorylation of CgAtg32 before mitophagy was also detected in iron-depleted C. glabrata cells (Fig. 1C) as observed in S. cerevisiae under nitrogen-starved conditions. These observations further support the existence of conserved autophagic/mitophagic machinery between 2 genetically related yeast species. The expression of genes indispensable for mitophagy, CgATG8 and CgATG32, was elevated at 4 h after the shift to iron-depleted conditions, dropped down at 8 h, and increased again during 24–48 h of incubation (Fig. S2A, B). The re-induction of ATG genes before 48 h may support the initiation of mitophagy on d 2; however, the role of the initially enhanced expression of the genes (at 4 h) is unclear. The sudden decrease in mRNA of the ATG genes at 8 h is curious. The reduction of mRNA was only partly suppressed in the autophagy-deficient atg1D mutant (Fig. S2C, D). These results suggest that some autophagic degradation may participate in the reduction of mRNA at 8 h under iron-depleted conditions; however, the rapidly increased cell number during the growing phase or the spontaneous mRNA degradation may also contribute to the reduced cellular mRNA of the ATG genes.

In S. cerevisiae, mitophagy was induced: (i) in nonfermented long-term culture, or (ii) short-period exposure to nitrogen-starved conditions after preculturing in nonfermentable medium.

Figure 5. Loss of mitophagy results in decreased intracellular ROS. Wild-type, Cgatg32Δ, and Cgatg32Δ CgATG32 cells were cultivated for 3 d under the indicated growth conditions. The mean ROS levels (A) and the ratio of ROS-positive cells (B) were determined by flow cytometric analyses of cells treated with dihydroethidium. The mean ± SEM values are representative of 3 independent experiments. Asterisks indicate statistically significant differences (*, P < 0.05). ns indicates no significant difference (P > 0.05). (C) Wild-type and Cgatg32Δ cells were cultured in SD, SD-Fe, or SD-Fe. After the indicated incubation period, oxidized proteins were detected by an OxyBlot assay.
Figure 6. Mitophagy is required for maintenance of mitochondrial membrane potential. Wild-type, Cgatg32Δ, and Cgatg32Δ CgATG32 cells were cultivated for 3 d under the indicated growth conditions. The mean MMP levels (A) and the ratio of MMP-positive cells (B) were determined by flow cytometric analyses of cells treated with DiOC6(3). The mean values ± SEM are representative of 3 independent experiments. Asterisks indicate statistically significant differences (*, P < 0.05). ns indicates no significant difference (P > 0.05).

Figure 7. ATG32 is required to maintain the fungal burden in mouse tissues. (A, B) Fungal tissue burdens in the kidney (A) and spleen (B) from groups of 5 BALB/c mice infected via the tail vein with $1 \times 10^7$ viable cells of C. glabrata strains. The results are expressed as CFU/g of tissue and they represent values recorded separately in each of the 5 mice. Geometric means are indicated by horizontal bars. Statistical comparisons are summarized above each panel. Asterisks indicate statistically significant differences (*, P < 0.05). NS indicates no significant difference (P > 0.05). (C) Immuno-suppressed CD-1 mice were inoculated with the C. glabrata wild-type strain CBS138. Mice were rendered neutropenic via intraperitoneal administration of cyclophosphamide (200 mg/kg of body weight per day) and cortisone acetate (125 mg/kg of body weight per day) 3 d before challenge and on the day of infection. Mice were injected with $1 \times 10^6$ viable cells. After 7 d, the mice were sacrificed and their kidneys were excised. Yeast present in excised kidneys were collected by pipette and transferred to a 1.5-mL collecting tube on ice. The collected cells were used in the expression analyses. The expression of CgATG32 is represented as the relative fold change compared with wild-type cells incubated under aerobic conditions at 30°C for 4 h. The values represent the mean and standard deviation based on triplicate measurements from a representative experiment.
induced mitophagy in *C. glabrata* alone. These results suggest that the regulatory mechanisms of mitophagy are partly conserved but different between the 2 yeast species. The difference in the mitophagy-inducing conditions for these 2 yeasts may be explained by variations in their usual growth environments and evolutionary gene rearrangements. Comparative genome analyses of *S. cerevisiae* and *C. glabrata* indicate that *C. glabrata* has lost genes involved in galactose, phosphate, nitrogen, and sulfur metabolism via genome evolution from the common ancestor of *S. cerevisiae*. As a result of these gene losses, *C. glabrata* exhibits auxotrophy for some nutrients such as nicotinic acid, pyridoxine, and thiamine.28,29 *C. glabrata* is thought to have undergone this reductive evolution to suit the host environment, like other pathogenic microorganisms.30 It is possible that *C. glabrata* has lost the genes responsible for mitophagy induction in response to short-period nitrogen starvation and it may have gained another mitophagic regulation system to survive inside the host’s body. *C. glabrata* is assumed to regulate mitochondrial function by mitophagy to survive in an iron-deficient environment, such as a host’s body. In iron-deficient conditions, both heme biosynthesis and respiratory activity could be inhibited.25,26 Therefore, in *C. glabrata*, it is expected that dysfunctional mitochondria are degraded by mitophagy to maintain mitochondrial homeostasis. Along these lines, PINK1-PARK2-independent mitophagy was reported in human osteosarcoma or neuroblastoma cells under iron-depleted conditions.31 Thus, iron depletion may be the common initiation signal for mitophagy among some eukaryotes.

Mitophagy is also responsible for the prolonged life span of *S. cerevisiae* during caloric restriction.11 In a mitophagy-deficient mutant strain (atg32Δ) of *S. cerevisiae*, which exhibits shortened CLS, the ROS level is increased whereas MMP is decreased compared with those in the WT.3 In terms of the relationship between life span and ROS, 2 opposite effects of ROS have been proposed in *S. cerevisiae*.12,16,32–34 It has been reported that ROS accumulation after the deletion of FLX1, which catalyzes the movement of the redox cofactor FAD across the mitochondrial membrane, shortens the life span.35 By contrast, Mesquita et al. reported that increased intracellular ROS levels due to the inactivation of Cta1 or Ctt1, the 2 main ROS-scavenging enzymes, extend the CLS.12 They also demonstrated that the decreased intracellular ROS caused by the overexpression of CT1 results in a shortened CLS.12 The discrepancy above could be partly illustrated by the “mitochondrial ROS signaling;” ROS are generally thought to shorten life span by bringing about oxidative damage on DNA or essential enzymes required for cell division; however, the elevated ROS level during the growing phase enhances oxidative stress responses, increases the intracellular enzymes that detoxicate ROS during stationary phase, and result in elongated CLS.36 In regard to MMP, it was reported that a reduction in the MMP in old cells or in the presence of dinotrophenol, a mitochondrial uncoupler, leads to a decreased life span.37–39 Thus, it is considered that MMP is maintained to support the intracellular ATP levels, thereby retaining the longevity of *S. cerevisiae*.

In the present study, we found that the disruption of CgATG32 reduced the life span of *C. glabrata* cells under iron-depleted conditions as in the case of *S. cerevisiae* under caloric-restricted condition (Fig. 4A). The MMP in *C. glabrata* was lower than that in WT cells (Figs. 6A, B, S6A–D) as expected from the result of *S. cerevisiae*, whereas the ROS in the mutant was lower than that in WT (Figs. 5A, B, S5A–D). In the case of *C. glabrata* grown under iron-depleted conditions, it is possible that the decreased ROS in Cgatg32Δ cells during the growing phase, failed to evoke mitochondrial ROS signaling, and resulted in the decreased CLS. These findings also indicate that mitophagy during the growing phase may be necessary for keeping active respiration in mitochondria and for induction of mitochondrial ROS signaling before entering stationary phase. However, these hypotheses need to be examined by further study with *C. glabrata* cells under other growing conditions or by other ROS-detection reagents.

We also found that supplementation of SD-Fe medium with N-acetyl-L-cysteine (NAC), a potential ROS scavenger, restored the decreased CLS of Cgatg32Δ cells to the level of WT cells (Fig. S7A). This result prompted us to hypothesize that the restored CLS of Cgatg32Δ by NAC was due to scavenging of ROS; however, the ROS level of Cgatg32Δ was less than that of WT cells under iron-depleted conditions as mentioned above (Fig. 5A, B). Moreover, NAC treatment did not decrease ROS levels or protein oxidation of either WT or Cgatg32Δ cells (Fig. S8A–C). Decreased MMP in Cgatg32Δ cells was also unaffected by NAC treatment (Fig. S9). In terms of ROS scavenging, the effect of NAC on improved CLS of Cgatg32Δ is completely unexplained; however, it is possible that NAC scavenged a kind of minor ROS, which are not detected in this study but are nonetheless harmful to replication.

NAC treatment also suppressed mitophagy in *C. glabrata* grown under iron-depleted conditions (Fig. S7B, C). NAC stimulates GSH synthesis after its conversion into cysteine, and is a mitophagy inhibitor in *S. cerevisiae*.40 Supplementation with N-acetyl-D-cysteine, an optical isomer of NAC with similar ROS scavenging properties to NAC but that does not promote GSH synthesis, does not inhibit mitophagy.40,41 Direct supplementation of GSH monoethylester in yeast cells successfully inhibits mitophagy.40 These previous results with *S. cerevisiae* suggest that the promotion of GSH synthesis may inhibit mitophagy during NAC treatment without the reduction of cellular ROS level. It has also been reported that CLS is increased by pharmacological inhibition of the synthesis of glutathione using L-buthionine-sulfoximine.12 Thus, there may be a relationship between longevity and regulation of the GSH content.

In mice infection models with *C. glabrata*, we found that the deletion of CgATG32 resulted in decreased kidney and spleen fungal burdens (Fig. 7). Since the expression of CgATG32 was increased in the host’s kidney, mitophagy is expected to be induced during infection and it is involved in the survival of the host tissue when depleted of free iron ions. The functions of mitochondria are thought to be inhibited by low oxygen and iron concentrations inside the host’s body, so mitochondria may be less necessary during infections than in aerobic growth conditions. Another possible physiological role of mitophagy in *C. glabrata* cells may be the degradation of unnecessary mitochondria to replenish the amino acid pool.
Most pathogens that can potentially cause bloodstream infections are thought to have developed highly efficient iron acquisition systems, including siderophores, low molecular weight organic chelators with a high affinity for Fe³⁺, to survive inside the host’s body. C. glabrata does not appear to possess the ability to synthesize siderophores or to efficiently utilize heme as an iron source. This indicates that the iron deficiency response of this fungus differs from that of other pathogenic microorganisms.

Mitophagy is thought to be involved in controlling the quantity and quality of mitochondria in S. cerevisiae, however the physiological importance of mitophagy was still unclear. Our results in this study support the idea that mitophagy is necessary for intact pathogenicity of C. glabrata cells probably through maintaining mitochondrial functions during infection. This study is for the first time proposing a physiological significance of mitophagy in a eukaryotic unicellular microorganism. Our results may also contribute to understanding the general role of mitophagy in higher eukaryotic organisms.

Materials and methods

Ethics

All our animal experiments were in compliance with the guidelines and policies of the Principles of Morality for Animal Experiments of the National Institute of Infectious Disease, Japan (approval number 114118-2).

Strains and growth media

Escherichia coli DH5α (F-, φ80, lacZΔM15, Δ (lacZYA-argF) U169, hsdR17 (k-r – mK+), recA1, endA1, deoR, thi-1, supE44, gyrA96, relA1 λ-) was used for plasmid propagation. Bacterial strains were grown in LB with 50 µg/mL ampicillin. The growth media for yeast were as follows: YPD (1% yeast extract, 2% peptone, 3% glucose, vitamins), SD (0.67% yeast nitrogen base, 2% lactate, pH 5.5), YPG (1% yeast extract, 2% peptone, 3% glucose; for nitrogen-starvation). Yeast strains and plasmids used in this study are listed in Table 1.

Construction of CgATG gene-disrupted C. glabrata strains

The DNA fragment used to replace the CgATG32, CgATG1, or CgATG11 ORF with CgHIS3 was amplified from the plasmid pHIS916 using the primer sets ATG32DF and ATG32DR (ATG1DF and ATG1DR, or ATG11DF and ATG11DR, respectively). Each amplified fragment (approximately 1 kb) was used to transform KUE200. Disruption of the CgATG genes was confirmed by PCR using primers pTET12F and ATG32CHR for CgATG32, pTET12F and ATG1CHR for CgATG1, and pTET12F and ATG11CHR for CgATG11, respectively. The sequences of all the primers are listed in Table S2. Integration of DNA fragments into the CgATG32 gene locus was also confirmed by Southern blot analysis (Fig. S1).

Table 1. Strains and plasmids used in this study.

| Strain          | Genotype or feature*                        | Reference |
|-----------------|--------------------------------------------|-----------|
| CBS138          | ATCC type culture                          |           |
| KUE200          | trpΔ his3Δ::URA3 ura3Δ FRT-YKU80            |           |
| Cgatg22Δ        | trpΔ his3Δ::URA3 ura3Δ FRT-YKU80 arg32Δ::HIS3 | This study|
| Cgatg11Δ        | trpΔ his3Δ::URA3 ura3Δ FRT-YKU80 ang1Δ::HIS3 | This study|
| Cgatg32Δ CgATG32| trpΔ his3Δ::URA3 ura3Δ FRT-YKU80 arg32Δ::HIS3-TRP1 | This study|
| Cgatg32Δ 3HA-CgATG32 | trpΔ his3Δ::URA3 ura3Δ FRT-YKU80 arg32Δ::HIS3-3HA-ATG32-TRP1 | This study|
| KUE200-mtDHFR-GFP | trpΔ his3Δ::GPDp-mito-mouse DHFR-GFP ura3Δ FRT-YKU80 | This study|
| Cgatg11Δ-mtDHFR-GFP | trpΔ his3Δ::GPDp-mito-mouse DHFR-GFP ura3Δ FRT-YKU80 arg32Δ::HIS3 | This study|
| Cgatg32Δ-mtDHFR-GFP | trpΔ his3Δ::GPDp-mito-mouse DHFR-GFP ura3Δ FRT-YKU80 arg32Δ::HIS3 | This study|
| BY4741          | MATa his3Δ leu2Δ mating type ura3Δ          |           |
| BYmtDHFR-GFP    | BY4741 [p416GPD-mtDHFR-GFP]                |           |

| Plasmid          | Feature                                      | Reference |
|------------------|----------------------------------------------|-----------|
| p416GPD-mtDHFR-GFP | CEN URA3 GPDp N. crassa ATP9 (1–69) + mouse DHFR + brighterGFP | 8         |
| pHIS906          | Carrying CgHIS3 marker, PCR template for the cassette amplification | 42        |
| pTi-comp         | Carrying CgTRP1 marker, PCR template for the cassette amplification | 48        |

* The indicated genes refer to C. glabrata unless otherwise noted. Sc; Saccharomyces cerevisiae.
**Reintroduction of CgATG32 into Cgatg32Δ mutants**

DNA fragments harboring the CgATG32 ORF were amplified from the CBS138 genome using the primers ATG32 compF/-1kb and ATG32 compR/1979r. The amplified fragment containing the CgATG32 ORF was digested with BamHI and XhoI, which yielded a DNA fragment that was cloned into the BamHI and SalI sites of pTi-comp. Using these plasmids as the template, insertion fragments were amplified using the primers ATG32-Rev-F and ATG32-Rev-R. The resulting DNA fragment was introduced into the CgHIS3 gene-replaced locus in the CgATG32 deletant chromosome by end-in type recombination. Accurate insertion of the amplified fragment into the correct chromosomal locus was confirmed by PCR using the primers ATG32 up −1050 to −1031 and ATG32 ORF 20–1. The sequences of all the primers are listed in Table S2. Integration of DNA fragment into the designated locus was also confirmed by Southern blot analysis (Fig. S1).

**Southern blot analysis**

Southern blot analysis was performed as described previously. All of the restriction enzymes used in the experiments are shown in Figure S1. The probe, which corresponded to nucleotides 200–480 or 44–606 relative to the start codon of CgHIS3 or CgTRPI, was amplified with the oligonucleotides HIS3f and HIS3r, or TRP1f and TRP1r, respectively (Table S2).

**Semi-quantitative RT-PCR**

Semi-quantitative RT-PCR was performed as described previously. All of the primers used for qRT-PCR are listed in Table S2. All experiments were repeated based on 3 independent preparations of RNA and the mean ± SEM represented the results of 3 experiments.

**Detection of mitophagy by western blotting**

Detection of mitophagy was conducted according to previously described methods. C. glabrata cells expressing HA-CgATG32 were cultured in 1 mL of YPD medium overnight. These cells were collected by centrifugation and washed twice with sterile water, inoculated at approximately 3×10^6 cells/mL, and cultured in 50 mL of SD or SD-Fe medium. The incubated cells were harvested at specific time points and the optical density at 600 nm (OD_{600}) was estimated. Cell aliquots equivalent to 30 OD_{600} unit were placed in 2.0 mL homogenizing tubes (Yasui kikai Co., ST-0250). These cells were collected by centrifugation and washed twice with ice-cold sterile water. The following procedures were performed on ice or at 4°C. The cells were suspended in 700 μL of homogenizing buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride [PMSF; Roche Applied Science, 1873636]), and 700 mg of glass beads (Sigma-Aldrich, G8772) were added to the cell suspension. The cells were disrupted using a Multi-beads shocker (Yasui kikai Co., MB1001[S]) at 2,700 rpm for 5 min. The cell extract was collected, and the glass beads were washed with up to 1 mL of homogenizing buffer containing 1 mM PMSF. The cell extract was centrifuged (2,000 × g for 10 min) to remove unbroken cells and cellular debris, and the supernatant fraction was centrifuged at 20,000 × g for 45 min. The pellet was washed with 500 μL of NEBuffer for PMP (50 mM Tris-HCl, pH 7.5, 2 mM DTT, 0.01% Brij 35 [New England Biolabs, P0753S], pH 7.5) and centrifuged at 20,000 × g for 45 min. The pellet was resuspended in 50 μL of NEBuffer for PMP with 1.5 μL of λPP (+; NEB, 400,000 U/ml) and without λPP (-), incubated at 30°C for 1 h and centrifuged at 20,000 × g for 45 min. The pellet fraction was resuspended in 30 μL of sample buffer and incubated at 100°C for 3 min. The protein concentrations of these samples were determined by a Bradford Protein Assay (APRO SCIENCE, KY-1030) with bovine serum albumin as the standard. Next, 20 μg aliquots of the samples were loaded onto a 10% polyacrylamide gel and resolved. A standard semi-dry western blot transfer procedure was performed using PVDF membranes. After blotting, the membranes were probed with anti-GFP antibody (1:25,000 dilution; Clontech, 632380) by incubating for 1 h at room temperature. After washing the membranes 3 times each for 10 min in Tris-buffered saline containing Tween 20 (TTBS; 50 mM Tris-HCl, pH 7.6, 0.9% NaCl, 0.1% Tween 20 [Sigma-Aldrich, P9416]), a secondary incubation was performed with HRP-conjugated anti-mouse IgG (1:20,000 dilution; Thermo Fisher Scientific, 32430) for 1 h at room temperature. After washing the membranes 3 times each for 10 min in TTBS, the GFP signal was detected using an ECL kit (Wako Pure Chemical Industries, Ltd., 296–69901). Quantification of the fluorescence intensity was performed using a C-DiGit Blot Scanner and ImageStudio software (LI-COR Biosciences, Lincoln, NE). MtDHFR-GFP and processed GFP were detected as bands that migrated at molecular masses of approximately 50 and 28 kDa, respectively.

**Lambda protein phosphatase (λ PP) treatment**

C. glabrata cells expressing HA-CgATG32 were cultured in 1 mL of YPD medium overnight. These cells were collected by centrifugation and washed twice with sterile water, inoculated at approximately 3×10^6 cells/mL, and cultured in 50 mL of SD or SD-Fe medium. The incubated cells were harvested at specific time points and the optical density at 600 nm (OD_{600}) was estimated. Cell aliquots equivalent to 30 OD_{600} unit were placed in 2.0 mL homogenizing tubes (Yasui kikai Co., ST-0250). These cells were collected by centrifugation and washed twice with ice-cold sterile water. The following procedures were performed on ice or at 4°C: the cells were suspended in 700 μL of homogenizing buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride [PMSF; Roche Applied Science, 1873636]), and 700 mg of glass beads (Sigma-Aldrich, G8772) were added to the cell suspension. The cells were disrupted using a Multi-beads shocker (Yasui kikai Co., MB1001[S]) at 2,700 rpm for 5 min. The cell extract was collected, and the glass beads were washed with up to 1 mL of homogenizing buffer containing 1 mM PMSF. The cell extract was centrifuged (2,000 × g for 10 min) to remove unbroken cells and cellular debris, and the supernatant fraction was centrifuged at 20,000 × g for 45 min. The pellet was washed with 500 μL of NEBuffer for PMP (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij 35 [New England Biolabs, P0753S], pH 7.5) and centrifuged at 20,000 × g for 45 min. The pellet was resuspended in 50 μL of NEBuffer for PMP with 1.5 μL of λPP (+; NEB, 400,000 U/ml) and without λPP (-), incubated at 30°C for 1 h and centrifuged at 20,000 × g for 45 min. The pellet fraction was resuspended in 30 μL of sample buffer and incubated at 100°C for 3 min. The protein concentrations of these samples were determined by a Bradford Protein Assay (APRO SCIENCE, KY-1030) with bovine serum albumin as the standard. Next, 20 μg aliquots of the samples were loaded onto a 10% polyacrylamide gel and resolved. A standard semi-dry western blot transfer procedure was performed as described above. Primary antibody treatment was performed using anti-HA antibody (F-7, 1:1,000 dilution; Santa Cruz Biotechnology, sc-7392).
Anaerobic incubation

Anaerobic conditions were obtained with an anaerobic rectangular jar and Anaero pack-Anaero (Mitsubishi Gas Chemical Company, Inc., A-41). C. glabrata strains were inoculated from an overnight saturated YPD culture to OD_{600} = 0.002 in 75 mL Flask with Vent Cap (Corning, 430641) with 30 mL SD medium at 30°C in the anaerobic jar with Anaero pack-Anaero.

CLS assay

Overnight cultures in 1 mL of YPD medium (starting from single isolated colonies) were diluted to 0.1 OD_{600} units in 10 mL of medium and incubated at 30°C with shaking at 200 rpm. Viability was measured by plating aging cells onto YPD agar plates and monitoring the CFU levels at specific time points. The initial viability was defined as 100%. The experiment was performed in triplicate.

Detection of protein oxidation level

Protein samples were prepared as described above for the western blotting analyses. The protein oxidation levels were determined using an OxyBlot™ Protein Oxidation Detection kit (Millipore, S7150), according to the manufacturer’s instructions.

Measurements of cellular ROS level and MMP

Overnight cultures in 1 mL of YPD medium (starting from single isolated colonies) were diluted to 0.1 OD_{600} unit in 10 mL of medium and incubated at 30°C with shaking at 200 rpm. The cells were collected by centrifugation and incubated in PBS with 15 μM DHE (Sigma-Aldrich, D7008) to measure the cellular ROS level or 175 nM DiOC₆(3) (Thermo Fisher Scientific, D-273) to measure the MMP, for 30 min at 30°C with shaking at 200 rpm. Stained cells were washed and resuspended in PBS, and then analyzed using a fluorescence-activated cell sorter (BD FACSCalibur; BD Biosciences). The obtained data were analyzed using FlowJo software (Tree Star). The following parameters were used: FL1 for DiOC₆(3), FL2 for DHE, side scatter, and forward scatter. The experiment was performed in triplicate (n = 3) and 30,000 events were registered for each sample. Statistical analyses were performed using GraphPad Prism™ (GraphPad Software) via an unpaired t test with a significance level of P < 0.05.

Animal studies

The tissue fungal burden and expression analysis were performed as described previously. The male BALB/c mice 7 wk of age (Japan SLC, Inc.) and the male CD-1 mice 4 wk of age (Charles River Laboratories Japan, Inc.) were used for evaluation of the fungal burden and the gene expression, respectively. CD-1 mice were rendered neutropenic by intraperitoneal administration of cyclophosphamide and cortisone acetate. The mice were injected into their tail vein with saline suspensions of a C. glabrata strain (in a volume of 200 μL). After seven days, mice were sacrificed, and target organs (kidney and spleen) were excised aseptically. Organ homogenates were diluted and plated onto YPD containing streptomycin sulfate salt (Sigma-Aldrich) and penicillin G sodium salt (Sigma-Aldrich). Colonies were counted after a day of incubation at 37°C, and the numbers of CFU/g of organ were calculated. In the case of CD-1 mice, C. glabrata colonies on kidney were collected, and used for gene expression analysis as described above. Statistical analyses were performed using GraphPad Prism™ via an unpaired t test with a significance level of P < 0.05. Primers used for the expression analysis are listed in Table S2.

Abbreviations

CFU colony-forming unit
CLS chronological life span
DHE dihydroethidium
DHFR dihydrofolate reductase
DiOC₆(3) 3',3'-dihexyloxacarbocyanine iodide
ECL enhanced chemiluminescence
GFP green fluorescent protein
GSH glutathione
HA hemagglutinin
MMP mitochondrial membrane potential
NAC N-acetyl-L-cysteine
OD optical density
PMSE phenylmethane sulfonyl fluoride
ROS reactive oxygen species
RT-PCR reverse transcription-polymerase chain reaction
SD synthetic glucose medium
SEM standard error of the mean
TTBS Tris-buffered saline containing Tween 20
WT wild type
YPD yeast extract peptone dextrose
λ PP lambda protein phosphatase

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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