Autophagy-Inducing Factor Atg1 Is Required for Virulence in the Pathogenic Fungus Candida glabrata

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Autophagy-Inducing Factor Atg1 Is Required for Virulence in the Pathogenic Fungus Candida glabrata

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Candida glabrata is one of the leading causes of candidiasis and serious invasive infections in hosts with weakened immune systems. C. glabrata is a haploid budding yeast that resides in healthy hosts. Little is known about the mechanisms of C. glabrata virulence. Autophagy is a ‘self-eating’ process developed in eukaryotes to recycle molecules for adaptation to various environments. Autophagy is speculated to play a role in pathogen virulence by supplying sources of essential proteins for survival in severe host environments. Here, we investigated the effects of defective autophagy on C. glabrata virulence. Autophagy was induced by nitrogen starvation and hydrogen peroxide (H2O2) in C. glabrata. A mutant strain lacking CgAtg1, an autophagy-inducing factor, was generated and confirmed to be deficient for autophagy. The Cgatg1Δ strain was sensitive to nitrogen starvation and H2O2, died rapidly in water without any nutrients, and showed high intracellular ROS levels compared with the wild-type strain and the CgATG1-reconstituted strain in vitro. Upon infecting mouse peritoneal macrophages, the Cgatg1Δ strain showed higher mortality from phagocytosis by macrophages. Finally, in vivo experiments were performed using two mouse models of disseminated candidiasis and intra-abdominal candidiasis. The Cgatg1Δ strain showed significantly decreased CFUs in the organs of the two mouse models. These results suggest that autophagy contributes to C. glabrata virulence by conferring resistance to unstable nutrient environments and immune defense of hosts, and that Atg1 is a novel fitness factor in Candida species.

Keywords: autophagy, Atg1, Candida glabrata, virulence, reactive oxygen species

Abbreviations: ATG, autophagy-related; CFU, colony forming units; Cg, Candida glabrata; CTA1, catalase A 1; DC, disseminated candidiasis; DCF, dichlorofluorescein; DMEM, Dulbecco’s modified Eagle’s medium; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; GFP, green fluorescent protein; H2O2, hydrogen peroxide; IAC, intra-abdominal candidiasis; ORF, open reading frame; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ROS, reactive oxygen species; SC-trp, synthetic complete medium lacking tryptophan; SD-N, synthetic dextrose medium lacking nitrogen; SE, standard error; YPD, yeast extract peptone dextrose.
Shimamura et al. CgAtg1 Requirement for C. glabrata Virulence

GRAPHICAL ABSTRACT | The deletion of ATG1 disrupted autophagy function in C. glabrata, leading to increased sensitivity to hydrogen peroxide (H$_2$O$_2$) and nutrient starvation. These phenotypes were associated with decreased fitness and virulences in host environments.

INTRODUCTION

Autophagy is an evolutionarily conserved biological process in eukaryotes that involves the degradation of cytosolic molecules for recycling intracellular materials (Klionsky et al., 2016). Autophagy has several functions: supplying nutrients under starvation conditions, maintaining homeostasis and protein levels, clearing damaged and/or non-functional proteins, and preventing bacterial and viral infections. These functions contribute to cell longevity, development, and differentiation as well as tumor suppression (Jin et al., 2017; Chen et al., 2018; Hansen et al., 2018). In the induction of autophagy, autophagosomes, which are double-membrane cytosolic vesicles, are formed to incorporate target proteins. In the model yeast Saccharomyces cerevisiae, autophagosomes fuse with vacuoles, and proteins are degraded in the vacuoles.

Fungal pathogens also use autophagy to survive in a host environment with unstable nutrient supply (Khan et al., 2012). However, little is known about the contribution of fungal autophagy to their persistence and virulence in hosts. In addition, immune defenses such as macrophages generate ROS to kill infecting pathogens (Gonzalez-Parraga et al., 2003; Thorpe et al., 2004), but it has remained obscure whether fungal autophagy affects this oxidative stress response.

Candida species are a genus of opportunistic fungal pathogens that cause severe invasive infections in immunocompromised patients (Miceli et al., 2011). Candida glabrata is the second most common cause of candidiasis in humans (Roetzer et al., 2011). The genetic background of C. glabrata is closely related to that of S. cerevisiae. C. glabrata is a commensal yeast and capable of surviving in the host longer than other Candida species (Roetzer et al., 2011). We hypothesized that autophagy contributes to these functions in C. glabrata.

Autophagy has several subcategories. Macroautophagy covers a broad range of protein degradation processes that are mainly induced upon starvation (Suzuki, 2013). Pexophagy and mitophagy are specific to the degradation of damaged peroxisomes and mitochondria, respectively (Suzuki, 2013).

Recently, pexophagy and mitophagy have been suggested to be related to C. glabrata virulence (Roetzer et al., 2010; Nagi et al., 2016). In the present study, we analyzed macroautophagy.

Macroautophagy (hereinafter simply referred to as autophagy) is induced by Atg proteins in yeasts (Yorimitsu and Klionsky, 2005). Atg1 is a component of an Atg protein complex and is essential for autophagy induction (Wang and Kundu, 2017). Candida glabrata Atg1 (CgAtg1) is also predicted to be important for autophagy, because ATG genes are highly conserved between S. cerevisiae and C. glabrata. Here, we showed that C. glabrata autophagy is induced by nitrogen starvation and H$_2$O$_2$. The CgATG1-deleted mutant of C. glabrata exhibited deficient adaptation to starvation and H$_2$O$_2$ in vitro. An ex vivo experiment using mouse peritoneal macrophages demonstrated that the Cgatg1Δ strain was phagocytosed by macrophages and showed low viability. Autophagy was revealed to be important for C. glabrata survival in two mouse models of invasive candidiasis.

MATERIALS AND METHODS

Ethics Statement

Animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, DC, 2011) and all of the institutional regulations and guidelines for animal experimentation after pertinent review and approval by the Institutional Animal Care and Use Committee of Nagasaki University (approval number 1407281164-4).

Culture Conditions

C. glabrata was routinely cultured at 30°C in SC-trp (Dunham et al., 2015) or YPD agar [1% yeast extract, 2% peptone, 2% dextrose, and 2% Bacto agar (BD Biosciences, B242720)], unless otherwise indicated. SD-N [0.17% yeast nitrogen base without amino acids and ammonium sulfate (BD Biosciences, 233520)]
and 2% dextrose] was used for the nitrogen starvation condition (Budovskaya et al., 2004).

**Strain and Plasmid Construction**

*C. glabrata* strains, plasmids, and primers used in this study are listed in Tables 1–3, respectively. Sequence information of *C. glabrata* genes was obtained from the *Candida* genome database.

The *C. glabrata atg1*Δ strain was constructed using a one-step PCR-based technique as described previously (Miyazaki et al., 2010a, 2011). Briefly, an *CgATG1* deletion construct was amplified from pBSK-HIS using primers tagged with 100-bp sequences homologous to the flanking regions of the *CgATG1* ORF (*CgATG1-100F* and *CgATG1-100R*). *C. glabrata* parent strains were transformed with the deletion construct, and the resulting transformants were selected by histidine prototrophy (Miyazaki et al., 2011). Successful homologous recombination was verified by diagnostic PCR, and the absence of *CgATG1* mRNA expression was confirmed by real-time qRT-PCR (data not shown). Transformation of *C. glabrata* was performed using the lithium acetate protocol, as described previously (Cormack and Falkow, 1999).

pCgACT-CgATG1, in which *CgATG1* was expressed under the control of the *CgATG1* native promoter, was constructed as follows: a 3,781-bp DNA fragment containing the *CgATG1* promoter, ORF, and 3′-UTR was amplified using *CgATG1-F* (−596FL)-Sal and *CgATG1-R* (+356FL)-Kpn, digested with SalI and KpnI, and inserted into the SalI-KpnI site of pCgACT (Kitada et al., 1996). An *CgATG1*-reconstituted strain and its control strains were constructed by transformation of the *Cgatg1*Δ strain with pCgACT-CgATG1 and pCgACT, respectively. They were selected by tryptophan prototrophy and verified by qRT-PCR (data not shown).

1http://www.candidagenome.org

**TABLE 1 | C. glabrata strains used in this study.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>CBS138</td>
<td>Candida glabrata wild-type</td>
<td>Dujon et al., 2004</td>
</tr>
<tr>
<td>2001T</td>
<td>CBS138/trp1Δ</td>
<td>Kitada et al., 1995</td>
</tr>
<tr>
<td>wild-type</td>
<td>2001T containing pCgACT</td>
<td>Miyazaki et al., 2011</td>
</tr>
<tr>
<td>WT+GFP-CgATG8</td>
<td>2001T containing pCgACT-GFP-CgATG8</td>
<td>This study</td>
</tr>
<tr>
<td>WT+CgCTA1-ΔOE</td>
<td>2001T containing pCgACT-CgCTA1</td>
<td>Nishikawa et al., 2016</td>
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<tr>
<td>KUE200</td>
<td>CBS138/trp1Δ, his3Δ, trp1Δ, FRT-YKU80</td>
<td>Ueno et al., 2007</td>
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<tr>
<td>Cgatg1Δ</td>
<td>KUE200/atr1Δ::His3 containing pCgACT</td>
<td>This study</td>
</tr>
<tr>
<td>Cgatg1Δ+CgATG1</td>
<td>KUE200/atr1Δ::His3 containing pCgACT-CgATG1</td>
<td>This study</td>
</tr>
<tr>
<td>Cgatg1Δ+GFP-CgATG8</td>
<td>KUE200/atr1Δ::His3 containing pCgACT-GFP-CgATG8</td>
<td>This study</td>
</tr>
<tr>
<td>Cgatg1Δ+CgCTA1-ΔOE</td>
<td>KUE200/atr1Δ::His3 containing pCgACT-CgCTA1</td>
<td>This study</td>
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</table>

**TABLE 2 | Plasmids used in this study.**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>pBSK-HIS</td>
<td>pBluescript II SK+ containing <em>C. glabrata</em> HIS3 at the Xho I site</td>
<td>Miyazaki et al., 2010a</td>
</tr>
<tr>
<td>pCgACT</td>
<td><em>C. glabrata</em> centromere-based plasmid containing autonomously replicating sequence and <em>C. glabrata</em> TRP1</td>
<td>Kitada et al., 1996</td>
</tr>
<tr>
<td>pCgACT-CgATG1</td>
<td><em>C. glabrata</em> ATG1 promoter, ORF, and 3′-UTR were inserted into the BamHI-I-Sal I site of pCgACT</td>
<td>This study</td>
</tr>
<tr>
<td>pCgACT-GFP-CgATG8</td>
<td><em>C. glabrata</em> ATG8 promoter, N-terminally GFP-tagged ORF, and 3′-UTR were inserted into the BamHI-I-Sal I site of pCgACT</td>
<td>This study</td>
</tr>
<tr>
<td>pCgACTP</td>
<td><em>S. cerevisiae</em> PGK1 promoter and <em>C. glabrata</em> HIS3 3′-UTR were inserted into the Sac I-Kpn I site of pCgACT</td>
<td>Miyazaki et al., 2010a</td>
</tr>
<tr>
<td>pCgACTP-CgCTA1</td>
<td><em>C. glabrata</em> CTA1 ORF was inserted into the BamHI-I-Sal I site of pCgACT</td>
<td>Nishikawa et al., 2016</td>
</tr>
</tbody>
</table>

**Growth Curve Construction**

Logarithmic-phase *C. glabrata* cells were adjusted to 5 × 10^6 cells/ml and incubated in SC-trp broth at 37°C. The number of cells was counted at 2, 4, 6, 8, 24, and 48 h. Doubling times were calculated as previously described (Geber et al., 1995), except that the cells were counted using a hemocytometer instead of OD_600. The averages of the doubling times were obtained from four independent experiments.

**Spot Assay**

Spot assays were performed as described previously (Miyazaki et al., 2010b). Briefly, logarithmic-phase cells grown in SC-trp broth were harvested and adjusted to a concentration of 2 × 10^7 cells/ml. Serial 10-fold dilutions were prepared, and 5 μl of each
dilution was spotted onto agar plates and incubated at 30°C for 48 h, unless otherwise indicated.

**Immunoblotting**

Anti-GFP (Roche, 11814460001), anti-Pgk1 (OriGene EU, AP21371AF-N), anti-mouse IgG-hrp (GE Healthcare, NA931V), and anti-rabbit IgG-hrp (GE Healthcare, NA934V) antibodies were purchased. Logarithmic-phase cells were harvested and resuspended in PBS with 10 μM CM-H$_2$DCFDA (Thermo Fisher Scientific, C6827) and ROS. Cell-permeable CM-H$_2$DCFDA is not fluorescent; it reacts with ROS to produce fluorescent DCF. Logarithmic-phase cells were washed with PBS, resuspended in PBS with 10 μM CM-H$_2$DCFDA, and incubated at 30°C for 1 h. The cells were washed, resuspended in SC-trp with H$_2$O (Wako, 084-07441), tert-butyl hydroperoxide (Wako, 026-13451), menadione (Wako, 132-08132), or diamide (Sigma-Aldrich, D3648), and cultured at 30°C for 1 h. The cells were washed and resuspended in PBS, and fluorescence was measured with a PHERAstar FS multi-mode fluorescence microplate reader (BMG LABTECH, Offenburg, Germany) at 485 nm fluorescence excitation wavelength and 520 nm emission wavelength. The cell count of each sample was determined, and relative fluorescence intensity per cell was calculated.

**Measurement of Intracellular ROS Levels**

Intracellular ROS levels in *C. glabrata* were analyzed by measuring fluorescent DCF derived from the reaction of CM-H$_2$DCFDA (Thermo Fisher Scientific, C6827) and ROS. Cell-permeable CM-H$_2$DCFDA is not fluorescent; it reacts with ROS to produce fluorescent DCF. Logarithmic-phase cells were washed with PBS, resuspended in PBS with 10 μM CM-H$_2$DCFDA, and incubated at 30°C for 1 h. The cells were washed, resuspended in SC-trp with H$_2$O (Wako, 084-07441), tert-butyl hydroperoxide (Wako, 026-13451), menadione (Wako, 132-08132), or diamide (Sigma-Aldrich, D3648), and cultured at 30°C for 1 h. The cells were washed, resuspended in PBS, and fluorescence was measured with a PHERAstar FS multi-mode microplate reader (BMG LABTECH, Offenburg, Germany) at 485 nm fluorescence excitation wavelength and 520 nm emission wavelength. The cell count of each sample was determined, and relative fluorescence intensity per cell was calculated.

**Lifespan Assay**

Incubation of 2.5 × 10$^6$/ml cells was started in ultrapure water (Thermo Fisher Scientific, 10977-023) at 30°C. The cells were harvested at multiple time points and plated on YPD agar. The number of viable cells was determined by counting colonies after incubation for 36 h at 30°C, and the number of CFUs per ml was calculated.

**Macrophage Infection Assay**

To collect mouse peritoneal macrophages, peritoneal exudates were obtained from 8-week-old male BALB/c mice (Japan SLC, Shizuoka, Japan) by lavage 4 days after intraperitoneal injection of 2 ml of sterile 4% thioglycollate broth (BD Biosciences, 211716). After washing with DMEM, peritoneal macrophages were incubated in DMEM with 10% FBS and 100 μg/ml gentamycin (Nacalai Tesque, 11980-14) at 37°C under 5% CO$_2$. Macrophages of 1.5 × 10$^5$ cells were seeded in 24-well plates and incubated in DMEM with 10% FBS for 24 h. For infection assays, 2.5 × 10$^5$ *C. glabrata* cells were resuspended in DMEM, added to the macrophages, and incubated. The actual *C. glabrata* cell number was confirmed by plating the cell suspension on YPD agar. Two hours after infection, the infected macrophages were washed thrice with PBS to remove non-phagocytosed *C. glabrata* cells and incubated in fresh DMEM. At multiple time points post-infection, the infected macrophages were observed under a microscope (BZ-X700, Keyence, Osaka, Japan). Then, the macrophages were lysed in water, and phagocytosed *C. glabrata* cells were plated on YPD agar containing streptomycin sulfate salt (Sigma-Aldrich, S1277) and penicillin G sodium salt (Sigma-Aldrich, P3032). The number of viable *C. glabrata* cells was determined by counting colonies after incubation for 36 h at 30°C, and the number of CFU per well was calculated.

**Mouse Studies**

Mouse experiments were performed as described previously (Miyazaki et al., 2010a,b). Briefly, to prepare cells for injection, cultured *C. glabrata* cells were resuspended in sterile saline and adjusted to 5 × 10$^8$ or 1 × 10$^9$ cells/ml. The actual *C. glabrata* concentration was confirmed by plating the adjusted cell suspension on YPD agar. The tail veins or abdominal cavities of 8-week-old female BALB/c mice (Japan SLC) were injected with 200 μl suspensions of each *C. glabrata* strain. The mice

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**TABLE 3 | Primers used in this study.**

<table>
<thead>
<tr>
<th>Gene deletion</th>
<th>Sequence (5' to 3')</th>
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</thead>
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<td>CgATG1-100F</td>
<td>GTTATCCAAAGCAATATAGCATAAAGGTTGAGTTGCTAGTTAGTGTCCTTTAAATTAGTACTCTGAGATGAGCTCCC</td>
</tr>
<tr>
<td>CgATG1-100R</td>
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</tr>
<tr>
<td>CgATG8-upR</td>
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<tr>
<td>CgATG8-down771R</td>
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</tr>
<tr>
<td>GFP-F</td>
<td>AAAAGTCGACCCATGAGGTTAGCAGGTTC</td>
</tr>
<tr>
<td>GFP-R</td>
<td>AATACCACTCCCGGGAAAATGCTCTAAAGGTGAAAGTATAATTC</td>
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<td>CgATG8-upF</td>
<td>GAATGATGACTTCATCCATGGTTTGTACAATTCATCCATACCATG</td>
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<td>CgATG8-down1153R</td>
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<td>GFP-F</td>
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<td>GFP-R</td>
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<tr>
<td>CgATG8-F</td>
<td>ATGAGGTGTCATCGAAGCTTTTGTGAGGCTT</td>
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<tr>
<td>CgATG8-upR</td>
<td>CCGGGGAGTGGTATTGAATTTCTTGG</td>
</tr>
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were sacrificed 7 days post-injection, and the target organs were excised. The organs were homogenized, diluted, and plated on YPD agar containing streptomycin sulfate salt and penicillin G sodium salt. Colonies were counted after incubation for 36 h at 30°C, and the number of CFU per organ was calculated.

RESULTS

Atg1 Is Necessary for Normal Growth Rate, Survival, and Autophagy During Nitrogen Starvation and Oxidative Stress
The Cgatg1Δ strain showed slightly slower growth than the wild-type strain and the CgATG1-reconstituted strain under the SC-trp growth condition (Figure 1). Doubling times of the wild-type, Cgatg1Δ, and CgATG1-reconstituted strains in SC-trp medium were 1.173, 1.912, and 1.254 h, respectively. Spot assays revealed growth defects in the Cgatg1Δ strain under the nitrogen starvation condition and in the presence of H2O2 (Figure 2A). These phenotypes recovered to the wild-type levels by the reintroduction of intact CgATG1 into the mutant.

It remains to be established whether or not autophagy is induced by H2O2 in C. glabrata. The induction of autophagy by starvation and the necessity of CgAtg1 for autophagy also need to be confirmed, as reports of autophagy in C. glabrata are still few. In S. cerevisiae, GFP-Atg8 is known to be cleaved by autophagy, resulting in the release of GFP, which can be detected as a marker of autophagy (Shintani and Klionsky, 2004). GFP-CgAtg8 was expressed in C. glabrata from a transformed plasmid containing the CgATG8 native promoter followed by an N-terminally GFP-tagged CgATG8 ORF. GFP-Atg8 and GFP bands were specifically observed in GFP-Atg8-expressing strains, but not in the negative control strain containing an empty vector (Figure 2B). GFP bands gradually became more intense as a result of nitrogen starvation and H2O2 exposure in the wild-type strain, whereas no GFP bands were observed over the time-course in the Cgatg1Δ strain (Figure 2B), indicating that autophagy was induced in response to nitrogen starvation and H2O2 exposure in a CgAtg1-dependent manner in C. glabrata.

In addition to the spot assay, a lifespan assay was performed to examine the sensitivity of the Cgatg1Δ strain to the starvation condition using pure water. Compared to the wild-type strain, the Cgatg1Δ strain showed a rapid decline in viability within 2 h (Figure 3A). Changes in C. glabrata viability became gradual after 2 h, and the Cgatg1Δ cells died after 4 weeks, in contrast to the wild-type cells that remained viable at 4 weeks in water (Figure 3B). Overall, these results suggest that autophagy plays a role in the adaptation to starvation in C. glabrata.

Analyses of the Correlation Between H2O2 Sensitivity and Intracellular ROS Levels
Intracellular ROS levels were increased by H2O2 in all the strains tested (Figure 4A). The Cgatg1Δ strain had a higher ROS level than the wild-type strain and the CgATG1-reconstituted strain regardless of the presence or absence of H2O2. High ROS levels in the Cgatg1Δ strain were also observed after exposure to other ROS-generating agents, including tert-butyl hydroperoxide (an oxidant), medionadine (a superoxide-generating agent), and diamide (an inhibitor of hydroxyl radical metabolism by decreasing the glutathione pool) (Supplementary Figure S1). The elevations of ROS levels in the Cgatg1Δ strain by these three ROS-generating agents were mild (approximately 10- to 15-fold relative to the control), while H2O2 markedly elevated the ROS level in the Cgatg1Δ strain (over 60-fold relative to the control). The sensitivity of the Cgatg1Δ strain to H2O2 was eliminated by the overexpression of CgCTA1 (CaTalase A 1) that encodes a catalase involved in the metabolism of H2O2 (Figure 4B). Consistent with the spot assay, the intracellular ROS amount in the Cgatg1Δ strain decreased to the level of the wild-type strain by CgCTA1 overexpression (Figure 4C). These results suggest that autophagy affects intracellular ROS levels and resistance to H2O2 in C. glabrata. CgCTA1 overexpression partially rescued the growth rate of the Cgatg1Δ strain under the SC-trp growth condition (Figures 4D,E). Doubling times of the wild-type, CgCTA1-overexpressed wild-type, Cgatg1Δ, and CgCTA1-overexpressed Cgatg1Δ strains in SC-trp medium were 1.136, 1.032, 1.995, and 1.499 h, respectively.

CgATG1 Deletion Decreases Resistance of C. glabrata to Macrophages
Activated macrophages generate H2O2 for phagocytosis (Forman and Torres, 2001). In addition, nutrients are restricted in the engulfed state by macrophages. Autophagy in C. glabrata might act to resist the immune response. To assess this hypothesis, an ex vivo experiment was performed using macrophages collected from mouse abdominal cavities. C. glabrata strains were added to wells containing the peritoneal macrophages and co-incubated, and viable C. glabrata cells phagocytosed by the macrophages were counted. Growth of the Cgatg1Δ strain was suppressed during the 96-h co-incubation, whereas growth of the wild-type strain and the CgATG1-reconstituted strain was increased despite phagocytosis by macrophages (Figure 5A). During the 96-h period post-infection, the increase in the number of wild-type C. glabrata cells was remarkable, whereas Cgatg1Δ cells were hardly observed (Figure 5B). This suggests that autophagy is required for resistance to macrophages in C. glabrata.

CgAtg1 Is Important for C. glabrata Persistence in Hosts
In mouse experiments with C. glabrata, DC mouse models are used because C. glabrata frequently spreads in patients through blood flow. Macrophages mainly cause inflammation in the abdominal cavity, although they also function in the blood and subsequent organs. To observe C. glabrata intraperitoneal infection, a mouse model of IAC was also employed. Liver, spleen, and kidney are the typical organs examined in the DC mouse model. The pancreas is the predominant organ for C. glabrata infection in the IAC mouse model, while the kidney is retroperitoneal and has not yielded stable results in previous studies (Cheng et al., 2013, 2014) or in our preliminary experiments (data not shown). Decreases in the

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FIGURE 1 | Growth curves of C. glabrata strains. Logarithmic-phase C. glabrata cells were grown in SC-trp broth at 37°C and counted at multiple time points. Graphs show data for the first 8 h of culture (A) to focus on the log phase and 48 h of culture (B). C. glabrata strains: wild-type (blue circles), Cgatg1Δ (magenta triangles), and Cgatg1Δ+CgATG1 (CgATG1-reconstituted Cgatg1Δ, green squares). The means ± SE of four independent experiments are shown. Doubling times: wild-type, 1.173 h; Cgatg1Δ, 1.912 h; Cgatg1Δ+CgATG1, 1.254 h.

FIGURE 2 | CgATG1-deleted C. glabrata is sensitive to nitrogen starvation and H2O2. (A) Logarithmic-phase cells were serially 10-fold diluted, spotted onto agar plates of SC-trp, SD-N, and SC-trp with 13.3 mM H2O2, and cultured at 30°C for 48 h. (B) N-terminally GFP-tagged CgAtg8 was expressed in the wild-type strain and the Cgatg1Δ strain. Cells were cultured in SD-N (left) or SC-trp with 13.3 mM H2O2 (right) for the indicated hours. Protein was extracted and immunoblotted using anti-GFP antibody and anti-Pgk1 antibody as a loading control. The protein extract of the wild-type strain without GFP-Atg8 expression was loaded as a negative control.

number of CFU were observed in the liver and spleen of the DC mouse model (Figure 6A) and all organs examined in the IAC mouse model (Figure 6B) when the mice were colonized by the Cgatg1Δ strain. In the kidney of the DC mouse model, the number of CFU slightly decreased in the Cgatg1Δ strain without statistical significance (Figure 6A). These results suggest that autophagy positively affects C. glabrata viability in the host.

DISCUSSION

Research on autophagy as a driving force of virulence is still in its developing stages compared to fundamental studies in humans and S. cerevisiae (Klionsky et al., 2016). We examined autophagy in the pathogenic fungus C. glabrata by conducting in vitro, ex vivo, and in vivo experiments, and found that autophagy has positive effects on C. glabrata virulence.

In vitro, CgAtg1 was required for autophagy, and the Cgatg1Δ strain was sensitive to the nutrient starvation condition, suggesting that autophagy in C. glabrata is important for adaptation to starvation, which has been observed in other fungal species such as Candida albicans and Cryptococcus neoformans (Palmer et al., 2008; Yu et al., 2015). Furthermore, rapid cell death and long-term survival defects were noted in the Cgatg1Δ strain in the lifespan assay, suggesting that autophagy functions in both adaptation to sudden environmental changes and for persistence of C. glabrata in hosts.

In pathogenic fungi, autophagy has been indicated to be dispensable for C. albicans virulence, whereas it is required for the infection process of C. neoformans (Palmer et al., 2008). C. glabrata is also a commensal yeast, but its infection mechanism is different from that of C. albicans. C. albicans forms hyphae to invade across the host environment and to escape from nutrient-starved loci and macrophages (Desai, 2018). C. glabrata seems to require autophagy because it lacks the capacity to form...
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**FIGURE 3** | CgAtg1 is important for C. glabrata viability under nutrient starvation conditions. C. glabrata strains were incubated in pure water at 30°C, harvested at multiple time points, and cultured in YPD agar. Then, the number of CFU was counted. Graphs show data for the first 4 h (A) and over 28 days (B) of incubation. The means ± SE of three independent experiments are shown.

**FIGURE 4** | Analyses of intracellular ROS level with H$_2$O$_2$ and effect of catalase. (A) C. glabrata strains containing CM-H$_2$DCFDA were cultured in SC-trp with/without 13.3 mM H$_2$O$_2$ at 30°C for 1 h, and the fluorescence produced by the reaction of CM-H$_2$DCFDA and ROS in the cells was measured. Relative fluorescence intensity per cell was calculated, and the fluorescence intensity of the wild-type strain without H$_2$O$_2$ was defined as 1. The means ± SE of three independent experiments are shown. Statistical analyses were performed using two-tailed Student’s t-tests. *P < 0.01; NS, no significance (P > 0.05). (B–E) Spot assays (B), measurement of intracellular ROS levels (C), and growth curve construction (D,E) were performed as in Figure 2A (B), Figure 4A (C), and Figures 1A,B (D,E) to analyze the effect of CgCTA1 overexpression. C. glabrata strains: wild-type (blue), WT+CgCTA1-OE (purple), Cgatg1Δ (magenta), and Cgatg1Δ+CgCTA1-OE (cyan). (D,E) Doubling times: wild-type, 1.136 h; WT+CgCTA1-OE, 1.032 h; Cgatg1Δ, 1.995 h; Cgatg1Δ+CgCTA1-OE, 1.499 h.
FIGURE 5 | CgATG1-deleted C. glabrata is sensitive to phagocytosis by macrophages. Mouse peritoneal macrophages were infected with C. glabrata strains in DMEM supplemented with 10% FBS. (A) Macrophages including C. glabrata were harvested at 2, 48, and 96 h post-infection, lysed in water, and plated on YPD agar for CFU count. The means ± SE of three independent experiments are shown. (B) Microscopic images of macrophages infected with C. glabrata for 96 h. Bottom panels are enlarged images of magenta boxes in the respective upper panels. Growing C. glabrata cells were observed in the wild-type strain and the Cgatg1Δ strain, but rarely in the Cgatg1Δ+ CgATG1 strain. Bar: 500 µm (upper), 100 µm (bottom).

FIGURE 6 | CgATG1 deletion decreases C. glabrata viability in mouse models of disseminated and intra-abdominal candidiasis. Groups of 7 to 10 BALB/c mice were inoculated with 1 × 10^8 cells intravenously (A) or 2 × 10^8 cells intraperitoneally (B) of each C. glabrata strain. Target organs were excised 7 days after injection. The organ homogenates were plated on YPD agar for CFU count. Numbers of CFU from each organ are indicated for individual mice in the scatter plots. Each geometric mean is shown by a bar. Statistical analyses were performed using the Wilcoxon Mann–Whitney test. *P < 0.01; NS, no significance (P > 0.05). Representative data of two independent experiments are shown.

hyphae but adheres to many different objects for a long time (Timmermans et al., 2018). The functional mechanisms of autophagy in oxidative stress response remain to be elucidated. This study showed high intracellular ROS levels in the Cgatg1Δ strain, especially by H_2O_2 addition. CgCTA1 overexpression rescued the resistance of the Cgatg1Δ strain to H_2O_2; however, this result does not indicate that autophagy promotes the expression of CgCTA1. Rather, factors other than the expression level of CgCTA1 are thought to be the cause of the high ROS levels in the Cgatg1Δ strain, as ROS elevation was also induced by other ROS-generating agents that are not metabolized by Cta1 (Supplementary Figure S1). Indeed, the rescue of the growth rate of the Cgatg1Δ strain in SC-trp by CTA1 overexpression was only partial and not up to the level of the wild-type strain (Figures 4D,E). Cta1 is considered to reverse the defect of the Cgatg1Δ strain only for adaptation to H_2O_2, but not for the overall functions of autophagy. Multiple factors are involved in the ROS elevation, as intracellular ROS levels including H_2O_2 are changed by various stresses and regulatory factors, and autophagy affects a wide range of molecules.

In addition to the regulation of intracellular ROS, it is speculated that fungal autophagy might affect the repair of cellular damage caused by H_2O_2. The adaptation to H_2O_2 has been analyzed in pexophagy in mammals and plants (Lee et al., 2014; Zientara-Rytter and Subramani, 2016). Peroxisomes generate H_2O_2 and are self-oxidized. These damaged peroxisomes are degraded by pexophagy for recycling, which leads to efficient maintenance of functional and fresh peroxisomes and the cleaning of junk; this is called ‘quality control.’ Recent studies in mammals have suggested that macroautophagy is also activated by H_2O_2 (Filomeni et al., 2015; Yin et al., 2015; Nah et al., 2017). From previous reports and this study, autophagy is supposed to have a two-step function in the adaptation to H_2O_2 (Supplementary Figure S2). The first step is the metabolism of overall ROS, and the second is the quality
control of proteins and organelles injured by \( \text{H}_2\text{O}_2 \). Further investigations are needed to elucidate the precise molecular mechanisms in \( \text{C. glabrata} \) and other species.

The \( \text{CgAtg1}\Delta \) strain exhibited slightly impaired growth and high ROS levels in the SC-trp medium (Figures 1, 2A, 4A). These defects are speculated to be caused by cellular stresses generated under this control condition, whereas the wild-type strain might have adapted to the stresses through a basal level of autophagy. The high ROS levels in the \( \text{CgAtg1}\Delta \) strain in the absence of any exogenous ROS agents are considered to be caused by the defect in metabolism of endogenous ROS generated by \( \text{C. glabrata} \) basal activities such as respiration. The endogenous ROS may also be a cause of the slow growth of \( \text{CgAtg1}\Delta \) strain. Indeed, the doubling time of \( \text{CgAtg1}\Delta \) strain was shortened to some extent by \( \text{CTA1} \) overexpression, although it was not rescued to the level of wild-type strain (Figures 4D,E). Stimuli other than ROS such as excessive ions, high osmolarity, and high temperature were analyzed, but the \( \text{CgAtg1}\Delta \) strain was not sensitive to these stresses (Supplementary Figure S3).

The function of autophagy in the adaptation to starvation is supposed to enhance \( \text{C. glabrata} \) viability in hosts, as nutrient conditions are thought to be unstable in various host environments. In addition, the effect of autophagy on ROS metabolism and resistance to \( \text{H}_2\text{O}_2 \) in \( \text{C. glabrata} \) was predicted to contribute to its survival against ROS-generating immune defenses, such as macrophages. An \( \text{ex vivo} \) experiment using mouse peritoneal macrophages revealed low viability of the \( \text{CgAtg1}\Delta \) strain in the macrophages (Figure 5). This defect can be attributed to the decreased resistance of the \( \text{CgAtg1}\Delta \) strain to both nutrient starvation and to the \( \text{H}_2\text{O}_2 \) encountered when phagocytosed by macrophages.

\textit{In vivo} mouse experiments confirmed that autophagy is a ‘fitness factor’ in \( \text{C. glabrata} \). Differences between the wild-type strain and the \( \text{CgAtg1}\Delta \) strain are thought to originate from the basic growth rate, the resistance to insufficient nutrient environments, and the oxidative stress response to macrophages, as well as other effects of autophagy that were not addressed in this study. Both the IAC mouse model, which was employed to examine effect of peritoneal macrophages, and the DC mouse model showed attenuated viability of the \( \text{CgAtg1}\Delta \) strain. These results suggest that immune defense not only by macrophages but also by other monocytes in the blood and subsequently infected organs might contribute to the decreased CFU of the \( \text{CgAtg1}\Delta \) strain. However, the number of CFU from kidneys was not significantly different between the wild-type strain and the \( \text{CgAtg1}\Delta \) strain in the DC mouse model. Although the exact reason is unclear, one possible explanation is that renal macrophages concentrate to the tubulointerstitium (Xu and Shinohara, 2017), a compartment of the kidney, and might not respond effectively to \( \text{C. glabrata} \) cells that usually invade the vasculature and nephrons in the kidney. Renal macrophages have a role in host defense against \( \text{C. albicans} \) (Lionakis et al., 2013; Ngo et al., 2014), but this function may be dependent on the \( \text{Candida} \) species. \( \text{C. glabrata} \) infection does not strongly attract immune cells, including neutrophils, and thus rarely causes severe inflammation (Kasper et al., 2015). Nevertheless, the results suggest that the extent of influence of autophagy in \( \text{C. glabrata} \) is substantially different among infected organs and tissues.

Although our experiments \textit{in vitro} and \textit{ex vivo} suggested the possibility of its contribution to virulence, obvious differences in viability and health status were not observed between the mice infected with the wild-type and \( \text{CgAtg1}\Delta \) strains. From our present results \textit{in vivo}, \( \text{C. glabrata} \) autophagy is concluded to be a fitness factor for longer survival in hosts.

CONCLUSION

Atg1 has been broadly investigated in many eukaryotes and established as a key player in autophagy induction (Wang and Kundu, 2017). However, reports of autophagy and Atg1 focusing on virulence are limited, especially in \( \text{Candida} \) species. The current study demonstrated for the first time that Atg1 is required for autophagy in \( \text{C. glabrata} \) (Graphical Abstract). Persistence in hosts is a characteristic of \( \text{C. glabrata} \) infection. The \( \text{CgAtg1}\Delta \) phenotypes indicate that autophagy may play an important role in fitness and survival of this fungus in infected hosts. The detailed characteristics of CgAtg1, including functional mechanisms, modifications, and interactions with other factors, need to be elucidated in future studies.

AUTHOR CONTRIBUTIONS

SS and TM designed the project, conducted the experiments, analyzed the data, and prepared the manuscript. All authors reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00027/full#supplementary-material
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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