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Candida albicans autophagy, no longer a bystander: Its role in tolerance to ER stress-related antifungal drugs

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ABSTRACT

Autophagy is a degradation process involved in pathogenicity of many pathogenic fungi. However, its roles in *Candida albicans*, the leading fungal pathogen in human beings, remain to be detailed. Most recently, we found that endoplasmic reticulum (ER) stress-inducing conditions led to transcriptional up-regulation of *C. albicans* autophagy-related (*ATG*) genes, implying a possible link between autophagy and ER stress response in this pathogen. Using a series of *C. albicans ATG* mutants and autophagy reporting systems, we found that both treatment of ER stress-related drugs and loss of the ER calcium pump Spf1 promoted autophagic flux of Atg8 and Lap41 (a homologue of *Saccharomyces cerevisiae* Ape1), indicating that these conditions induce autophagy. Moreover, deletion of *ATG* genes in the *spf1*Δ/Δ mutant rendered cells hypersensitive to these drugs and caused activation of UPR, revealing a role of autophagy in alleviating ER stress. In addition, only treatment of tunicamycin and loss of Spf1 in combination increased autophagic flux of the ER component Sec63, suggesting that most of the ER stress-related conditions cause non-selective autophagy rather than selective ER phagy. This study uncovers the important role of *C. albicans* autophagy in ER stress response and tolerance to antifungal drugs.

1. Introduction

Autophagy is an evolutionarily conserved process in eukaryotic cells, by which cellular nutrient materials and damaged organelles are recycled to maintain nutrient homeostasis and organelle functions (Hale et al., 2013). Macroautophagy (hereafter termed autophagy), the most well-characterized and typical autophagic pathway, is hallmarked by the formation of double-membrane vesicles known as autophagosome, followed by transport of these cargo-contained vesicles into the vacuole or the lysosome and degradation of internal cargo for recycling (Munch et al., 2014; Suzuki et al., 2002; Inoue and Klionsky, 2010; Codogno and Meijer, 2005). This process is involved not only in nutrient reusing and clearance of aggregates, but also in resistance to environmental stress (Munch et al., 2014; Berangere et al., 2003; Levine and Klionsky, 2004). In particular, in many pathogenic fungi, such as *Magnaporthe oryzae* and *Cryptococcus neoformans*, autophagy plays an important role in pathogenicity and pathogen-host interactions (Bartoszewska and Kiel, 2011; Liu et al., 2007; Lu et al., 2009; Hu et al., 2008). However, this process was supposed to be dispensable for virulence of *Candida albicans* and *Aspergillus fumigates* (Bartoszewska and Kiel, 2011; Palmer et al., 2007; Richie et al., 2007).

Nowadays, the frequency of opportunistic fungal infections is on the rise, due to the growing population of immunocompromised patients and those individuals suffered from organ transplantation, chemotherapy or antibiotic abuse (LaFayette et al., 2010; Gudlaugsson et al., 2003; Wisplinghoff et al., 2004). In this worldwide pathogen community, *C. albicans* is the leading fungal pathogen. It causes not only superficial mucosal infections, but also life-threatening systemic candidiasis (Pfaller and Diekema, 2007; Echeverria et al., 2011). However, there are limited kinds of antifungal drugs, mainly azoles, which are applied in clinical practice for treatment of *C. albicans*-related infections (LaFayette et al.,

Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; ATG, auophagy-related; MM-N, nitrogen source-depleted minimum medium; Cvt, cytosol-to-vacuole; TEM, transmission electron microscopy; PM, plasma membrane; cER, cytoplasmic ER; AP, autophagosome; ERA, ER-containing autophagosome; ERAD, ER-associated degradation.

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2010; Cowen and Steinbach, 2008). With the emergence of drug resistant strains, it is essential and urgent to explore the mechanisms by which this pathogen responds to and survives in the treatment of these drugs.

In fungal cells, the ER plays pivotal roles in lipid synthesis, protein folding, modification and secretion in all eukaryotic cells, all of which are required for biosynthesis of the essential structure, the cell wall (Lecomte et al., 2003; Tsvetanova, 2012; Görlach et al., 2006; Mora-Montes et al., 2007). Thanks to the significance and functional specificity of the ER in fungal cells, this organelle becomes the main target of antifungal agents. Most of them, such as tunicamycin and azoles, lead to ER stress in these organisms (Scrimale et al., 2009; Bonilla and Cunningham, 2003; Bonilla et al., 2002). As a well-known response mechanism, unfolded protein response (UPR) is activated, resulting in up-regulation of UPR genes to alleviate this stress (Bonilla et al., 2002). In *C. albicans*, UPR activation is mediated by its essential transcription factor, Hac1, and this activation plays an important role in resistance to ER stress-related antifungal agents, such as tunicamycin and dithiothreitol (DTT). However, the *hac1*Δ/Δ mutant is viable under treatment of the antifungal drugs, although it shows increased sensitivity to the agents (Wimalasena et al., 2008). This indicates that there must be other mechanisms by which the fungal cells respond to and survive in ER stress.

Previously, we identified an important ER P-type Ca^{2+}-ATPase in *C. albicans*, which mediates cytoplasmic Ca^{2+} sequestration into the ER. Deletion of its encoding gene, *SPF1*, causes disturbance of ER calcium homeostasis and ER dysfunction, suggesting that this deletion, similar to treatment of ER stress-related drugs, may also cause ER stress (Yu et al., 2012, 2013). Interestingly, transcription profiling we most recently performed demonstrated that expression of several *ATG* genes was up-regulated in the *spf1*Δ/Δ mutant, even without treatment of antifungal drugs (our unpublished data). This finding led us to provide a hypothesis that ER stress caused by treatment of the antifungal drugs or *SPF1* deletion may activate autophagy, which then plays a role in withstanding this stress and mediating cell survival.

Therefore, in this study, we identified abundant autophagy-related (*ATG*) genes, and investigated the possible induction effect of antifungal drugs and *SPF1* deletion on autophagy. We here demonstrate that, as expected, *SPF1* deletion together with ER stress-related drugs activated *C. albicans* ER phagy-like autophagy, leading to up-regulation of *ATG* genes, and enhanced autophagic flux. Moreover, dysfunction of autophagy in the *spf1*Δ/Δ mutant showed increased sensitivity to antifungal drugs and macrophage attacks, and had reduced ability of killing the host cells. Taken together, these findings revealed that *C. albicans* autophagy plays an important role in alleviating ER stress and is involved in virulence of this pathogen.

2. Materials and methods

2.1. Plasmids

Plasmids used in this study are listed in Table S1. For generating the reconstituted plasmids of *ATG1* and *ATG8*, the *ATG1* reconstituted cassette (containing the 2505 bp of open reading frame plus 1000 bp of promoter sequence and 300 bp of terminator sequence) and the *ATG8* reconstituted cassette (containing the 492 bp of open reading frame plus 708 bp of promoter sequence and 200 bp of terminator sequence) was amplified from the genome of the wild-type *C. albicans* strain BWP17 (Wilson et al., 1999), and then cloned into pDDB78, obtaining the reconstituted plasmids pDDB78-ATG1 and pDDB78-ATG8, respectively.

The Atg8-localization plasmid pAU34M-GFP-ATG8 was constructed as follows. Firstly, the *ATG8* fragment containing its terminating code was amplified from the described genome, and then was N-terminally fused with GFP fragment obtained from the plasmid pGFP-URA3 using the PCR method. The fusion fragment was digested by *Xho* I/*BamH* I, and then inserted into pAU34M, obtaining the localization plasmid pAU34M–GFP-ATG8.

To generate the UPR reporting plasmids P_{PMT4}-GFP and P_{PRB1}-GFP, and the *ATG* reporting plasmids P_{ATG1}-GFP, P_{ATG5}-GFP and P_{ATG8}-GFP, the promoters of *PMT4* (633 bp), *PRB1* (877 bp), *ATG1* (805 bp), *ATG5* (990 bp) and *ATG8* (708 bp) were amplified from the described genome, and then inserted into the plasmid pGFP (Dantas et al., 2010; Barelle et al., 2004), obtaining the corresponding UPR reporting plasmids and *ATG* reporting plasmids.

2.2. Strains

C. albicans strains used in this study are listed in Table S2. All *C. albicans* strains were generated in the background of the wild-type strain BWP17. The single-gene mutants, such as *atg1*Δ/Δ, *atg2*Δ/Δ and *atg8*Δ/Δ, were constructed by PCR-mediated homologous integration (Wilson et al., 1999). As an example, for disrupting the *ATG8* gene, BWP17 was transformed with the *ARG4* cassette amplified from the plasmid pRS-ArgΔ*Spe*I, generating the heterozygous mutant NKF158. NKF158 was then transformed with the *URA3* cassette amplified from the plasmid pDDB57, obtaining the homozygous mutant NKF159. To facilitate the following genetic manipulation, the homozygous mutant was plated on SC agar (2% glucose, 0.67% yeast nitrogen base, 0.2% amino acid mixture, 2% agar) containing 0.1% 5-FOA (BBI, USA), generating the *URA3*-depleted strains NKF160 (*atg8*Δ/Δ). For constructing the *ATG8* reconstituted strain, the homozygous mutant NKF160 was transformed with the *Nru* I-digested reconstituted plasmid pDDB78-ATG8, obtaining NKF161 (*ATG8c*).

The *spf1*Δ/Δ *atg8*Δ/Δ double mutant, in which both *SPF1* and *ATG8* were disrupted, was constructed in the *spf1*Δ/Δ (NKF111) background. Firstly, the *URA3*-depleted *spf1*Δ/Δ mutant (NKF162) was obtained by plating the NKF111 cells on the 5-FOA-contained SC agar, and then transformed with the *URA3* cassette amplified from the plasmid pDDB57, generating the *ATG8* heterozygous mutant (NKF163), followed by *URA3* depletion, obtaining heterozygous NKF164. This strain was then transformed again with the *URA3* cassette, generating the *ATG8* homozygous mutant NKF165. Finally, NKF165 cells were also plated on the 5-FOA-contained SC agar, obtaining the *URA3*-depleted double mutant NKF166 (*spf1*Δ/Δ *atg8*Δ/Δ).

To construct Atg8 localization strains, BWP17, *atg1*Δ/Δ, *spf1*Δ/Δ and *ATG1c* were transformed with the *Bgl* II-digested plasmid pAU34M–GFP-ATG8, obtaining the strains NKF167 to NKF170. To detect the aminopeptidase Lap41, BWP17, *spf1*Δ/Δ, *atg8*Δ/Δ, *spf1*Δ/Δ *atg8*Δ/Δ and *ATG1c* were transformed with the GFP-URA3 fragment amplified from the plasmid pGFP-URA3, which was inserted into the 3′ terminus of the *LAP41* gene, obtaining the strains NKF171 to NKF175. The ER membrane protein Sec63 was also C-terminally tagged by GFP with the same method, obtaining the strains NKF176 to NKF179.

For evaluating the effect of gene deletion or treatment of ER stress-related drugs on expression of UPR genes or *ATG* genes, the UPR reporting plasmids and the *ATG* reporting plasmids were digested by *Stu* I, and then transformed into *C. albicans* cells, obtaining the strains NKF180 to NKF195 for GFP fluorescence assays.

In order to eliminate the effect of *URA3* expression levels on virulence in systemic infection assays and phagocytic assays, the *URA3*-depleted strains were transformed with the *Pst* I- and *Bgl* II-digested plasmid pLUBP (Brand et al., 2004), generating the *in situ URA3*-reconstituted strains NKF196 to NKF199.

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