CHAPTER SEVENTEEN

Autophagy in the Filamentous Fungus *Aspergillus fumigatus*

Daryl L. Richie* *and* David S. Askew*

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Abstract

Many breakthroughs in our understanding of the function and molecular basis of autophagy have been achieved in mammalian and yeast systems. However, we still know very little about the contribution of autophagy to the biology of filamentous fungi. A comparative analysis of autophagy between genera will expand our knowledge of the autophagy machinery and has the potential to identify novel functions that are relevant to multiple biological systems. This chapter will discuss methods that have been employed for studying autophagy in the opportunistic mold pathogen *Aspergillus fumigatus*. Understanding how autophagy influences the growth of this important human pathogen could lead to the development of novel antifungal drugs that restrict the growth of the fungus by manipulating the autophagy pathway.

* Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, USA

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1. Introduction

Aspergillus fumigatus is a filamentous fungus that resides in compost and decaying vegetation. Human encounters with this mold are practically unavoidable because the organism is ubiquitous and releases high concentrations of conidia (spores) into the atmosphere after physical disturbance of its environment (Goodley *et al.*, 1994; Hospenthal *et al.*, 1998; Woodcock *et al.*, 2006). The inhalation of these conidia is generally innocuous in a healthy individual but can be life-threatening in patients with depressed immunity (Maschmeyer *et al.*, 2007). Because current antifungal drugs have been unable to prevent the high mortality rate associated with invasive aspergillosis (Upton *et al.*, 2007), there is a need for increased understanding of fungal pathways that could be targeted with novel therapy. One possible target that has been largely unexplored in this fungus is the autophagy machinery. As the major pathway for bulk turnover of organelles and other cytoplasmic constituents, active autophagy is likely to be incompatible with rapid fungal growth and would therefore be a promising drug target if further research was able to identify ways to selectively induce fungal autophagy through pharmacological intervention (Mizushima *et al.*, 2008).

In *A. fumigatus*, autophagy is required for conidiation and hyphal foraging, both of which are adaptive responses to nutrient deficiency that are important to the survival of the organism in its native environment (Richie *et al.*, 2007). Recent data have also suggested that autophagy contributes to metal ion homeostasis in *A. fumigatus*, but the mechanism by which this is accomplished requires further investigation (Richie *et al.*, 2007). Similarly, questions remain about the importance of autophagy to fungal virulence. For example, although autophagy is required for the virulence of some eukaryotic pathogens (Besteiro *et al.*, 2006; Hu *et al.*, 2008; Liu *et al.*, 2007; Veneault-Fourrey *et al.*, 2006), it is dispensable for the virulence of others (Palmer *et al.*, 2007), including *A. fumigatus* (Richie *et al.*, 2007). Thus, a comprehensive understanding of how autophagy influences the virulence of some, but not all, eukaryotic pathogens, may provide insight into mechanisms of eukaryotic pathogenesis. Because autophagy in eukaryotic pathogens is an emerging field of investigation, it becomes important to adapt current methodologies to the unique physiology of these organisms. This chapter outlines protocols that have been used to examine autophagy and autophagy-dependent processes in *A. fumigatus*.

2. Analysis of Autophagy-Dependent Processes in *A. fumigatus*

Analysis of autophagy in *A. fumigatus* has identified three quantifiable processes that depend on autophagy for optimal activity: starvation foraging, conidiation, and growth under metal ion–deficient conditions. Assays for

these processes can therefore be used as a qualitative indicator of autophagy function when evaluating autophagy mutants, with the caveat that autophagy is not the only pathway that can influence these functions.

2.1. Starvation foraging

The mycelium of a filamentous fungus comprises a network of interconnected hyphae that are separated by perforated septa. When the mycelium exhausts its nutrient supply, the organism is able to take advantage of these interconnections to support a limited amount of growth at the colony periphery, even though the rest of the colony is starved (Prosser and Tough, 1991; Robson, 1999). This foraging-like behavior allows the colony to expand into regions of unexplored substrate and has been hypothesized to involve the recycling of cytoplasmic contents by autophagy (Shoji *et al.*, 2006). To test this, an assay was developed to monitor the foraging capacity of *A. fumigatus* (Fig. 17.1). Hyphal plugs containing portions of mycelium are transferred to water/agarose (WA) starvation medium and colony diameter is monitored daily as a measure of starvation foraging. The ability of *A. fumigatus* to grow in this assay requires the Atg1 kinase (Richie *et al.*, 2007), which suggests that autophagy-controlled recycling of endogenous nutrients supports the foraging of the hyphal tips under acute starvation conditions. The autophagy dependence of this assay, and its ease-of-use, makes it a convenient adjunct to more sophisticated analyses of autophagic activity.

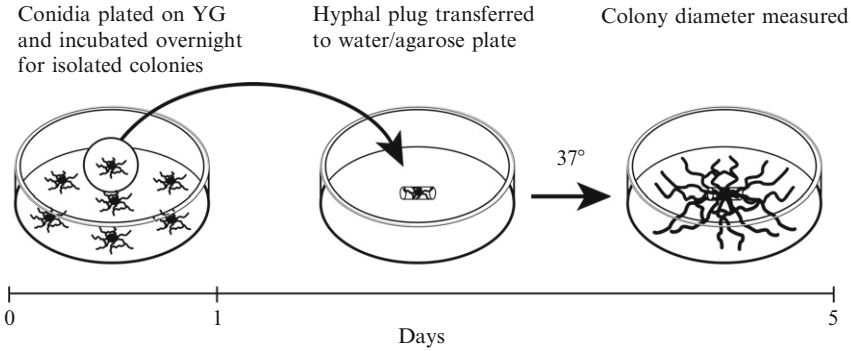

Figure 17.1 Schematic representation of the procedure used to monitor starvation-associated foraging in *A. fumigatus*. Hyphal plugs are transferred from rich medium onto WA lacking any nutrients, thereby forcing the organism to use autophagy to fuel any further growth. Colony diameter is measured daily as a relative indicator of active autophagy.

2.1.1. Media

YG plates: 2% glucose, 0.5% yeast extract, 1.5% Bacto agar. Combine ingredients in sterile distilled water, autoclave, and cool in a 50 °C water bath prior to pouring plates.

WA plates: Add 1g of agarose (genetic analysis grade) to 100 ml of Millipore ultrapure water in a clean 250 ml Erlenmeyer flask, autoclave, cool to 50 °C in a water bath, and pour 10 ml into 60-mm Petri dishes. Note: Bacto agar cannot be used in these plates because it contains sufficient nutrients to support the growth of an autophagy mutant.

2.1.2. Assay

1. *A. fumigatus* lacks a known sexual stage, but produces abundant asexual conidia (spores) on most types of laboratory medium. To harvest the conidia, flood the plate with 20 ml of sterile distilled water and dislodge the conidia by gently rubbing the top of the colony with a sterile Q-tip.
2. Aspirate the liquid with a 25-ml pipette, and remove the hyphal debris by filtration with sterile Miracloth into a 50-ml conical centrifuge tube (Calbiochem cat. 475855). Miracloth is a rayon-polyester material with a typical pore size of 22–25 μm.
3. Pellet the conidia by centrifuging at 4200*g* for 5 min at 4 °C. Wash twice with 50 ml of sterile distilled water and resuspend in 10 ml of sterile distilled water.
4. The spherical conidia are 2–3 μm in diameter and can be counted microscopically using a standard hemacytometer. Adjust the concentration to 1×10^4 conidia/ml.
5. Spread 20 μl of the 10^4 conidia/ml conidial stock solution (200 conidia) onto the surface of a 60-mm YG plate using a glass rod or hockey stick to evenly distribute the conidia.
6. Incubate at 37 °C for 24 h.
7. Using a five and three-fourth inch Pasteur pipette plugged with cotton wool, remove a hyphal plug from the edge of an individual colony on the YG plate and expel the plug onto the center of a WA plate using a small bulb.
8. Incubate at 37 °C for 4–6 days.
9. Measure the diameter of the colony starting with the first measurement after 24 h of incubation. The extent of growth after 24 h provides a baseline for further measurement because it is the same in both wild type (wt) *A. fumigatus* and an autophagy mutant (probably as a result of the carryover of nutrients from the original plug of YG agar). However, subsequent growth is autophagy dependent, which results in radial extension of very thin hyphal filaments. Because hyphal density is much lower on WA medium than rich medium, colony diameter is best monitored with a dissecting scope (Fig. 17.2). This is accomplished by drawing a horizontal line through the colony on the back of the plate

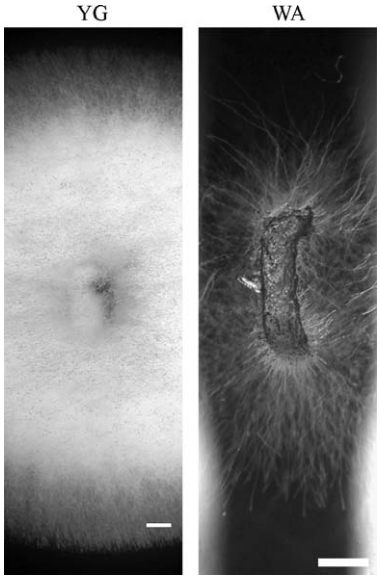

Figure 17.2 Hyphal density is reduced on WA starvation medium. An agar plug containing wt *A. fumigatus* was isolated from a plate of rich medium (YG) using a Pasteur pipette and transferred to a plate of YG or WA and incubated at 37 °C for 24 h. Colony size and hyphal density are greatly reduced on WA relative to YG, although the hyphae continue to expand radially for up to 5 days on WA. Colony morphology was photographed using a Nikon SMZ1000 dissecting microscope. Scale bar represents 10 mm.

on day 1 and marking the subsequent migration of the hyphal tips along this line with continued incubation.

Note: The ability to grow under these conditions is strain dependent. The wt strains H237 and CEA17 work well, but Af293 is less effective. However, this can be alleviated by transferring more Af293 biomass to the WA plates. The reason for this difference is not known.

2.2. Conidiation

In filamentous fungi, the production of conidia requires the construction of new morphological structures that are responsible for packaging nuclei into spores. Current evidence suggests that autophagy is essential for this process, presumably by providing the building blocks that support the necessary developmental changes (Richie *et al.*, 2007).

2.2.1. Media

Potato-dextrose agar (PDA): 1% glucose, 2% potato flakes, and 1.5% Bacto agar. Combine all ingredients in sterile distilled water, autoclave, and cool in a 50 °C water bath prior to pouring into 60-mm Petri plates.

2.2.2. Assay

1. Harvest conidia as described in section 2.1.2 and resuspend in sterile distilled water at a concentration of 1×10^6 conidia/ml.
2. Spot 5 μl of the 1×10^6 conidia/ml stock (5000 conidia) onto the center of triplicate PDA plates and incubate at 37 °C for 3 days.

Note: wt *A. fumigatus* conidiates extensively on PDA medium, but an autophagy mutant requires supplementation of the medium with increased nitrogen to support the same level of conidiation. Ammonium tartrate, ammonium chloride, ammonium sulfate, or sodium nitrate can be added to autoclaved PDA medium to a final concentration of 40 m*M* to restore conidiation to wild-type levels.

3. To harvest conidia, add 10 ml of sterile distilled water to each plate and gently rub the surface of the plate with a sterile Q-tip. Aspirate the water from the plate with a 10-ml pipette, and filter through Miracloth to remove hyphal fragments.
4. Repeat step 2 three times to maximize conidial recovery.
5. Count the total number of conidia recovered from each of the triplicate plates using a hemacytometer. For the wt strain a typical dilution of 1:100 will provide a countable number of conidia; autophagy mutants will usually require a lower dilution.

2.3. Growth in metal-depleted medium

The hypersensitivity of an *A. fumigatus* autophagy mutant to ethylene diamine tetraacetic acid (EDTA) suggests that the autophagy machinery contributes to the maintenance of metal-ion homeostasis in this fungus, possibly by recycling metals from preexisting metal-associated proteins (Richie *et al.*, 2007). This hypersensitivity can be quantified by inoculating conidia into liquid cultures containing EDTA and measuring percentage germination.

1. Place a sterile coverslip into a 60-mm Petri dish and overlay with 5 ml of liquid YG medium in the presence or absence of 0.5–0.75 m*M* EDTA.
2. Harvest conidia as described in section 2.1.2 and resuspend in sterile distilled water at a concentration of 1×10^6 conidia/ml.
3. Inoculate the YG-EDTA medium with 200 μl of the 1×10^6 conidia/ml stock solution and incubate at 37 °C for 3–6 days.
4. Remove the coverslip, and count the number of germinated conidia microscopically. The germination of *A. fumigatus* conidia involves a series of morphological changes, beginning with a short period of isotropic growth (swelling) that is followed by the establishment of an axis of polarity and the extension of the first germ tube. In this study, a conidium was scored as germinated if it had extended a germ tube that was equal to, or greater than the length of the conidium.

3. Analysis of Autophagosome Accumulation

After the induction of autophagy and the fusion of the autophagosome to the vacuolar membrane, a single membrane vesicle termed an autophagic body is delivered into the vacuole lumen (Mizushima *et al.*, 2008). Under normal circumstances, autophagic bodies are rapidly degraded by vacuolar hydrolases, making them difficult to visualize. However, their degradation can be delayed by incorporating the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) into the medium. This allows autophagic bodies to accumulate within the vacuole, and their appearance by light microscopy can be used as a qualitative marker of active autophagy (Klionsky *et al.*, 2007). To differentiate autophagic vesicles from other material that may accumulate in the vacuole, a second assay is recommended that involves fluorescent tagging of autophagosomes. This approach involves the creation of a strain of *A. fumigatus* that expresses a green fluorescent protein (GFP)-tagged Atg8 protein. Because a fraction of the Atg8 produced in a cell is continually associated with the autophagosome until it is finally degraded in the vacuole, the presence of GFP-Atg8 fluorescence can be used as a marker of autophagosome delivery into the vacuole (Fig. 17.3; Klionsky *et al.*, 2007).

3.1. GFP-tagging Atg8

1. Link the N terminus of the *A. fumigatus atg8* gene (GenBAnk accession No. Q4WJ27), or the corresponding cDNA, to GFP by standard cloning procedures in a plasmid vector. A 6-His linker can be used to separate GFP from the Atg8 protein (Richie *et al.*, 2007), but it is not known whether the linker is absolutely necessary. The plant-adapted GFP works

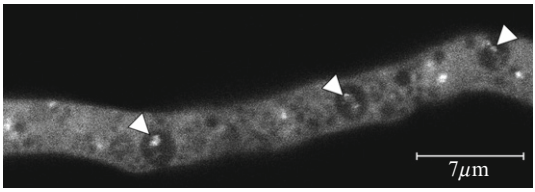

Figure 17.3 Analysis of autophagic body accumulation in starved cultures of *A. fumigatus*. Conidia from a strain expressing GFP-AfAtg8 were incubated in *Aspergillus* minimal medium for 16 h at 37 °C. The hyphae were washed and transferred to sterile distilled water (starvation conditions) containing 2 m*M* PMSF (to impair vacuolar protease activity). After incubating at 37 °C for 4 h, autophagic body accumulation in vacuoles was visualized by laser confocal microscopy. The image shown is a single optical section through a hypha. Arrowheads denote autophagic bodies in vacuoles. Scale bar represents 7 μm.

well in *A. fumigatus* (Bhabhra *et al.*, 2004), but other fluorescent tags can also be used, including ECFP and DsRed-Monomer (unpublished observations). The strong constitutive promoter *gpdA* has been successfully used to drive GFP-*atg8* from an ectopically integrated transgene in *A. fumigatus* (Richie *et al.*, 2007). However, techniques for tagging chromosomal genes with GFP have also been reported in *Aspergillus spp*, allowing expression levels to be under the control of the native promoter (Szewczyk *et al.*, 2006).

2. Digest 10 μg of the GFP-atg8 plasmid to linearize and introduce into the desired strain as an ectopic transgene by standard protoplast transformation as previously described (Bhabhra *et al.*, 2004).
3. The accumulation of GFP-labeled autophagic bodies within vacuoles can be visualized by fluorescence microscopy using the PMSF technique described subsequently.

3.2. Assay

1. Place a sterile glass coverslip into a 35-mm Petri dish
2. Harvest conidia as described in section 2.1.2, and inoculate 10,000–50,000 conidia into 5 ml of *Aspergillus* minimal medium. Incubate at 30–37 °C for 16 h.

Note: The incubation time and temperature can be adjusted to ensure that the culture has completely germinated. However, it is important not to overgrow to maintain synchrony between hyphal compartments (certain compartments become starved and begin to vacuolate prematurely when overgrown). In addition, overgrown cultures are more resistant to PMSF.

3. Remove the medium gently so as not to dislodge the mycelium from the coverslip.
4. Wash gently 3 times with sterile distilled water.
5. Remove the last of the distilled water and replace with 5 ml of starvation medium supplemented with 2 m*M* PMSF. The starvation medium can be water lacking any nutrients, or minimal medium lacking a nitrogen source. Note: PMSF precipitates in *Aspergillus* minimal medium at concentrations above 2 m*M*.
6. Incubate at 37 °C for 2–4 h. Extended incubation times cannot be used because the PMSF is toxic to the organism and results in cytoplasmic breakdown.
7. Aspirate the medium, wash the coverslip once with sterile distilled water, and mount the coverslip on a glass slide with the mycelium facing down.
8. Visualize autophagic bodies by fluorescence microcopy.

4. Conclusion

These methods have provided the first insight into the contribution of the autophagy kinase Atg1 to the growth and virulence of *A. fumigatus*. However, autophagy in filamentous fungi is still an emerging field that awaits adaptation of the many sophisticated techniques that have been developed in other systems. The high level of conservation of the autophagy machinery between genera underscores the importance of this pathway to eukaryotic cell biology, which makes continued investment in vigorous methods of analysis a high priority for future research.

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