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Vacuoles and fungal biology

Veronica Veses, Andrea Richards and Neil AR Gow

Fungal vacuoles have long been recognised as versatile organelles, involved in many aspects of protein turnover, cellular homeostasis, membrane trafficking, signalling and nutrition. Recent research has also revealed an expanding repertoire of physiological functions for fungal vacuoles that are vital for fungal growth, differentiation, symbiosis and pathogenesis. Vacuole-mediated long-distance nutrient transporting systems have been shown to facilitate mycelial foraging and long-distance communication in saprophytes and mycorrhizal fungi. Some hyphae of plant and human fungal pathogens can grow under severely nutrient-limited conditions by expanding the vacuolar space rather than synthesising new cytoplasm and organelles. Autophagy has been recognised as a crucial process in plant pathogens for the initiation of appressorium formation. These studies demonstrate the importance of fungal vacuoles as organelles that are essential for many of the attributes that define the activities and roles of fungi in their natural environments.

Addresses
The Aberdeen Fungal Group, School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom

Corresponding author: Veses, Veronica (v.veses@abdn.ac.uk), Richards, Andrea (arichards@abdn.ac.uk) and Gow, Neil AR (n.gow@abdn.ac.uk)

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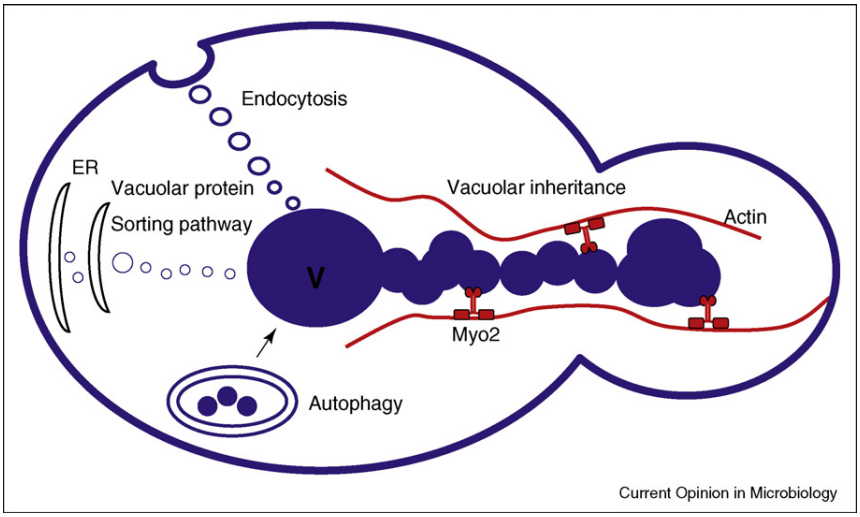
Introduction

Fungal vacuoles are acidic storage compartments with certain similarities to plant vacuoles and mammalian lysosomes. Most studies of fungal vacuoles have focused on its various functions, such as glycoprotein turnover and hydrolysis, the storage of Ca^{2+}, phosphate and amino acids, in pH and osmotic regulation, ion homeostasis and cytoplasmic detoxification [1,2]. *Saccharomyces cerevisiae* has served as a model to elucidate the mechanisms of vacuolar biogenesis, protein sorting, inheritance and the processes of vacuolar transport and homotypic membrane fusion and homeostasis (reviewed in references [3,4,5•,6]) (Figure 1). The vacuole is of significant interest to the membrane trafficking field since it receives membrane from biosynthetic, endocytic and autophagic pathways of the cell (Figure 1). Collectively, these studies have established the credentials of the fungal vacuole as a vital organelle at the heart of fungal physiology. Recent studies have also shown that vacuoles exist with a wide range of morphologies and that the roles of these vacuoles are highly adapted to the requirements of the ecological niche of specific species. They participate directly in long-distance nutrient transport through mycelia, in the regulation of hyphal extension and branching, and in cell-cycle timing and via autophagy they participate in the induction of vital morphogenetic processes such as appressorium formation and pseudohyphal growth. The objective of this review is to underline the importance of vacuole biology in the various lifestyles of important fungal species.

The vacuole and the fungal life style

The fungi are the second most diverse eukaryotic group, after the insects, and they are remarkable in the wide range of saprophytic, symbiotic and pathogenic life styles and cellular morphologies that they can adopt. Life style and cellular morphogenesis are often coupled; for example, species that are able to undergo yeast-hypha transitions include many human and plant pathogenic fungi [7] and host invasion often involves the induction of specialised penetration structures, such as appressoria. Filamentous growth of fungi is thought to be adapted to facilitate foraging, infiltration and ramification within natural environments to obtain fresh nutrients in a way that gives them an advantage over sessile unicellular organisms. In the extreme case of colonies of *Armillaria bulbosa*, now recognised as representing the world's largest and oldest living organisms, individual mycelia can occupy, feed and communicate over many hundreds of hectares of forest floor [8]. This begs questions about how the growth and activities of a mycelium can be coordinated over such long distances. Some basidiomycetes are capable of recycling the biomass from regions of mycelium that have depleted local nutrient supplies and redirecting this solubilised biomass through many centimetres or metres of mycelium to the foraging margin of the colony [9]. Mycorrhizal fungi can distribute nutrient resources not only within their mycelium, but also from plant to plant, to create cross-species network akin to a living 'motorway' transport system that links members of a dispersed community of interdependent individuals. Advanced real-time imaging techniques have been applied to the analysis of a range of fungal colonies and have shown extremely rapid distribution of soluble nutrients in different directions within mycelial networks

Figure 1



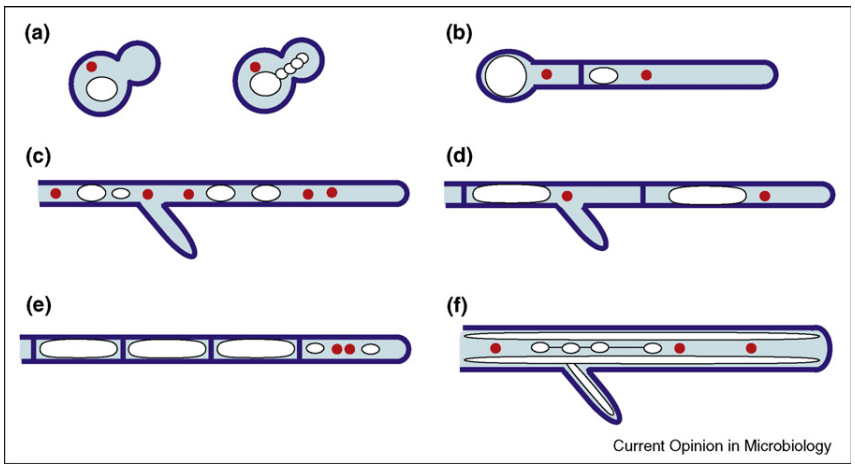
Convergent membrane trafficking involving the pathways for vacuole protein sorting, endocytosis, autophagy and vacuole inheritance in budding yeast. A stream of vacuole vesicles that form the vacuole segregation structure, which is mobilised by the Myo2-actin cytoskeleton is also shown.

[10•]. Emerging evidence places vacuoles at the centre of these transport networks enabling nutrient resources to be degraded, sorted, transported and redistributed. Other fungi are able to grow and explore their environments under highly oligotrophic conditions. For example leaf surfaces are often nutrient-limited, yet many plant pathogens are able to grow over these surfaces before they invade the plant via an appressorium. *Puccinia* and *Ustilago* species can grow on barren plant surfaces by minimising the biosynthetic costs associated with increasing their cytoplasmic volume. Both appressorium induction and growth under low nutrient conditions can be linked to important features of vacuole biology.

Vacuole inheritance and morphology

The ordered pathway of events that results in cell-cycle regulated inheritance of vacuoles has been dissected in *S. cerevisiae*. Yeast cells contain one to five vacuoles per cell [11] and these undergo a dynamic series of fission and fusion events throughout the cell-cycle. A wide range of mutants have been isolated and characterised that are interrupted in the processes of vacuole protein sorting

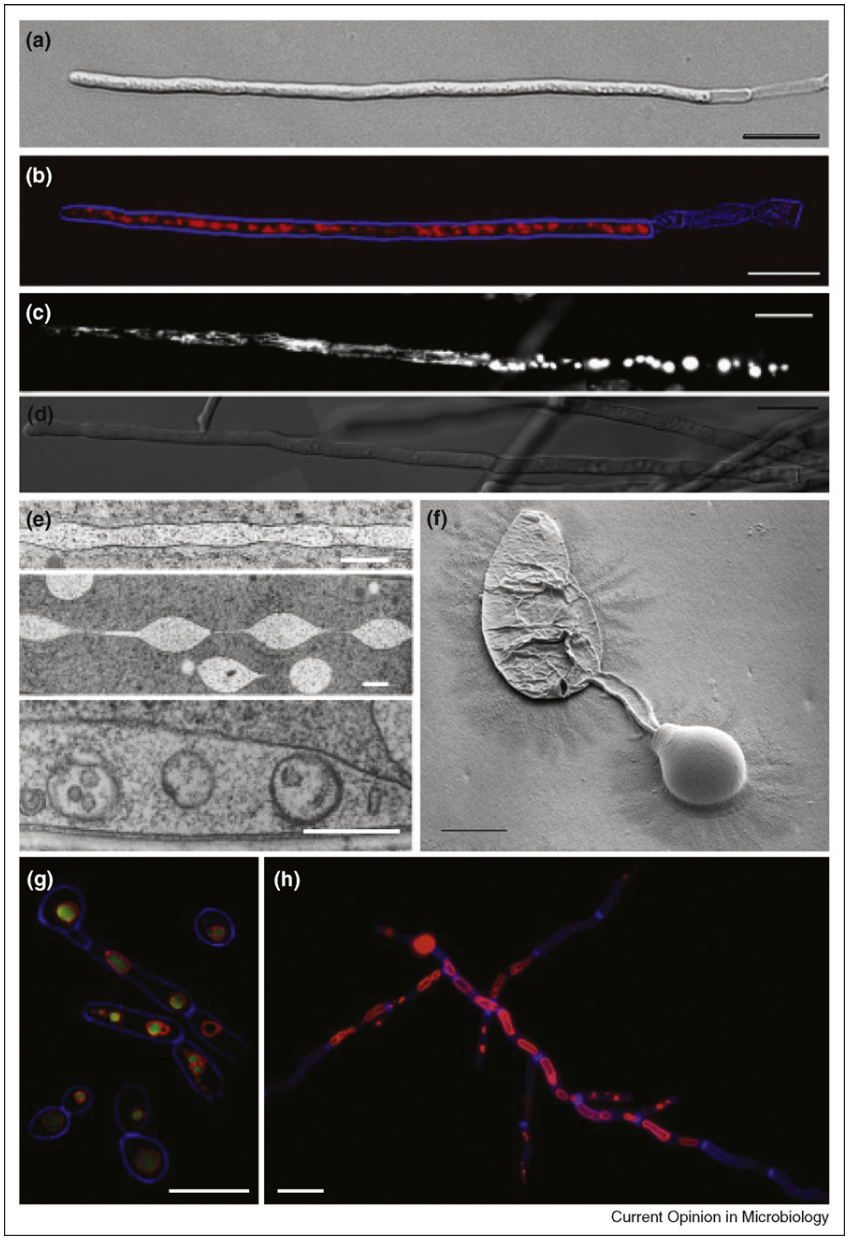
Figure 2



Vacuolar (white circles) and nuclear (red dots) organisation **(a)** in budding yeasts such as *Saccharomyces cerevisiae* and *Candida albicans*, **(b)** the true hyphal form of *C. albicans*, **(c)** a typical filamentous ascomycete, **(d)** *Basidiobolus ranarum*, **(e)** the plant pathogen *Ustilago maydis* and **(f)** tubular vacuole system typical of *Phanerochaete*, *Pisolithus* and many other fungi, as described in the text.

(*vps*), vacuole transport, homotypic membrane fusion and inheritance (reviewed in references [3,4,5•,12]) (Figure 1). A stream of small vacuoles or elongated tubular vacuoles is transported into the bud via an actin and Myo2-dependent transport system [5•]. In filamentous fungi there is also evidence of a role for dyneins in vacuole [13] and endosome motility [14]. The proportion of total cell volume occupied by vacuole differs substantially

Figure 3



Vacuole systems of fungi. **(a and b)** Vacuoles in 'empty' distal compartments of a hypha (black arrow heads) and cytoplasmic proximal dikaryotic hypha (white arrowheads) of *U. maydis*. Carboxypeptidase Y was fused to red fluorescent protein to visualise the vacuolar lumen (courtesy of G Steinberg). **(c and d)** Tubular vacuole system of a *Pisolithus* hypha imaged using differential interference contrast **(d)** and fluorescence using carboxy-CFFDA labelling **(c)** of the vacuole (courtesy of A Ashford and permission of Springer). **(e)** Transmission electron microscopy of tubular vacuoles in *Pisolithus* hyphae showing an elongated vacuole (upper panel), con-joined beads of vacuoles (middle panel) and a tubular vacuole adjacent to the cell wall with multivesicular inclusions (lower panel) (courtesy of A Ashford with permission from American Society of Plant Biology, The Company of Biologists and Elsevier, respectively). **(f)** Scanning electron micrograph of an empty autophagic conidium attached to the appressorium of *M. grisea* (courtesy of R Howard). **(g)** Vacuoles in yeast and pseudohyphal cells (**h**) and true hyphae of *C. albicans*. Cell walls were stained with Calcofluor White and vacuoles with CDC-FDA and FM4-64 (**g**) or FM4-64 alone **(h)** (images from V Veses). Scale bars represent 5 μm (a and b), 20 μm (c and d), 0.5 μm (e—all three panels) and 10 μm (g and h).

in different fungi and also differs between different cells of any given fungus. While many filamentous fungi have numerous small vacuoles dispersed in the cytoplasm, some have much larger vacuoles behind the apical region (Figures 2 and 3). Hyphal tips of ectomycorrhizal fungi and some other mycelial fungi contain an extensive reticulum of motile interconnected tubules and spherical vacuoles (Figure 3). Some fungal cells are extensively vacuolated and these highly expanded vacuoles can occupy almost the entire volume of the cell. Highly vacuolated regions usually occur in the distal regions of hyphae. In many plant pathogens vacuolated distal cell compartments are non-nucleated but in other fungi, such as the human pathogen *Candida albicans*, the distal hyphal compartments are nucleated (Figures 2 and 3).

Cellular homeostasis

Many of the transport, storage and homeostatic functions of the vacuole rely on ability to maintain an acidic lumen, through the action of the vacuolar ATPase [2,15]. Proton antiport systems exist for arginine, arginine-lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine-asparagine and isoleucine-leucine to enable amino acids to be accumulated at high concentrations [16]. The uptake of many ions is also energised by proton antiport. Ions, such as Cd^{2+}, Co^{2+} and Cu^{2+}, are potentially toxic, and storage in the vacuole results in detoxification of the cytosol [17,18]. The vacuole is also the main cellular location of storage for phosphate and polyphosphate, which occurs as chains of tens or many hundreds of residues (reviewed in reference [19]). Under phosphate-replete conditions, polyphosphate can form visible granules that exhibit Brownian motion within the vacuole. Polyphosphate acts as a calcium counter-ion and hydrolysis of polyphosphate leads to the release of protons, suggesting a role of polyphosphate in pH regulation [20].

The functional complexity of the vacuole is reflected in the diversity of membrane trafficking pathways that converge upon it [6,21]. Vacuole luminal and membrane associated proteins including proteases, aminopeptidases, α-mannosidase and alkaline phosphatase are transported through the secretory pathway, passing from the endoplasmic reticulum to the Golgi apparatus and then are diverted from the secretory pathway by the vacuolar protein sorting pathway (Figure 1). Material to be degraded in the vacuole can be delivered to the vacuole by endocytosis or the autophagy pathways [22,23] (Figure 1). The latter operates when massive degradation of cellular material is required, such as in response to stress or starvation [6,24–26] (Figure 1).

Tubular vacuoles: role in long-distance transport

A characteristic of mycelial fungi that differs from the vacuole biology of unicellular fungi is their highly pleiomorphic nature. In ectomycorrhizal fungi, hyphal tips contain an extensive reticulum of motile interconnected tubules and spherical vacuoles (reviewed in reference [27]) (Figures 2 and 3). The motile tubular vacuole system appears to be involved in long-distance transport of nutrients through the mycelial filaments [28,29]. In the ectomycorrhizal fungus *Pisolithus tinctorious*, the vacuoles transport nutrients between the plant and the fungal hyphal tips [30]. FRAP and photobleaching experiments have shown that the vacuoles in hyphae of *Phanerochaete velutina* are interconnected [31] and nutrient diffusion, perhaps aided by peristaltic-like movements of the vacuole, result in bidirectional movements of nitrogen and phosphate contained within them (reviewed in references [27,32]). In the vesicular arbuscular mycorrhizal fungus, *Gigaspora margarita*, it was shown that the tubular vacuoles did not follow the same paths as main bulk of the cytoplasmic flow suggesting that a combination of cytoplasmic flow plus tubular vacuolar transport was highly efficient in distributing nutrients within the fungus [33].

The presence of tubular vacuoles has also been reported in a non-ectomycorrhizal fungus, *Aspergillus oryzae*, by using a fusion protein of the putative t-SNARE, *Aovam3*, tagged with enhanced green fluorescent protein [34]. Studies of the movement of this fusion protein suggested that tubular vacuoles are formed extensively in hyphae that are not in direct contact with nutrients, in agreement with the idea that tubular vacuoles are involved in nutrient transport across different parts of the mycelia [35•].

A recent study of the intracellular trafficking of chitin synthases in *Neurospora crassa* showed that two chitin synthases, Chs3 and Chs6, accumulated in the lumen of a network of tubular and globular vacuolar compartments in distal regions of the *N. crassa* hyphae, before being delivered to the Spitzenkörper in the hyphal apex. This may even suggest the participation of the vacuolar system as an alternative route for the cytoplasmic transport of proteins that are destined for the cell surface [36].

Vacuoles and hyphal growth

Some filamentous fungi undergo extensive vacuolation behind the hyphal apex. Vacuole biogenesis is energetically less costly than the synthesis of new cytoplasm; therefore, hypha space-filling by vacuoles becomes an important aspect of nutrient budgeting in the ecology of many fungal species. The infection of maize by the basidiomycete *Ustilago maydis* requires the fusion of two haploid sporidia of different mating type to form the filamentous dikaryotic hypha. This invasive form of the fungus is able to induce tumours in meristematic tissues of the maize plant. Hyphal growth involves a cytoplasm-filled tip cell that does not undergo a mitotic cycle but extends and leaves behind empty-looking highly vacuolated and septate cell compartments [37]

(Figures 2 and 3). Growth of this dikaryotic hypha is therefore uncoupled from the cell-cycle so that growth is not balanced and extension occurs without the requirement for nuclear division.

Basidiobolus ranarum is an ascomycetous frog pathogen that also generates vacuolated, anucleate distal hyphal compartments. However, in this case, hyphal growth involves continuous expansion and forward migration of the apical cytoplasm. Nuclear division followed by cytokinesis and branch formation divides this compartment in two each time the cytoplasmic volume doubles [38].

In *C. albicans*, emergence of the germ tube involves substantial enlargement of the vacuole in the mother yeast cell while most of the cytoplasm migrates into the hypha [39]. In subsequent cell-cycles vacuole is again inherited asymmetrically at cytokinesis so that the distal compartments inherit most vacuole and the growing apical cell inherit most cytoplasm (Figures 2 and 3). In contrast to the distal vacuolated compartments of *U. maydis* hyphae, the extensively vacuolated subapical compartments of *C. albicans* are nucleated and are capable of forming a branch after a period of cell-cycle arrest [40•]. The observation that such highly vacuolated compartments are cell-cycle arrested suggests that the vacuole space may affect the timing of cell-cycle initiation and does this by influencing the threshold cytoplasmic volume required to execute the cell size-dependent G1 Start event. Highly vacuolated cell compartments are therefore arrested in G1 until sufficient cytoplasm is synthesised for them to trigger Start and renter the cell-cycle by forming a branch ([41,42] Veses *et al.*, unpublished). This pattern of growth associated with extensive vacuolation can again be interpreted as being an adaptation for growth under nutrient-depleted conditions.

Mutants of *C. albicans* that are affected in vacuole inheritance and translocation also have defects in hyphal development and in branching frequency ([41–49], Veses *et al.*, unpublished). Disruption of *PEP3/VPS18* in *Aspergillus nidulans* led to a pleiotropic phenotype, with fragmented vacuoles, clustered nuclei and mitochondria, and defects in the polarisation of the actin cytoskeleton [50]. Similarly, mutations in *VMA1*, which encodes a subunit of the vacuolar ATPase in *Neurospora crassa*, resulted in an irregular vacuolar morphology, with changes in vacuolar size that correlated with changes in branching frequency [51]. Thus, vacuole biology seems to play both direct and indirect roles in cell-cycle progression, hyphal growth and branch initiation in fungi.

Autophagy and cellular differentiation

In the rice blast fungus *Magnaporthe grisea*, germinating spores produce a polarised germ tube, which differentiate at the tip to form an appressorium [52,53]. The *M. grisea* appressorium generates up to 8 MPa of turgor to provide enough force to breach the cuticle of the rice plant [54••]. The vacuole undergoes extensive expansion to support appressorium formation and, through the degradation of lipid stores, generates osmotically active metabolites. This process increases the turgor pressure of the maturing appressorium to facilitate the forced entry of the fungal penetration peg into the plant epidermis [52,54••] (Figure 3). A second function for the vacuole during appressorium development is in its contribution to the autophagic cell death that is associated with the nuclear degeneration and collapse of the conidial spore [54••]. Appressorium formation and function is contingent on a developmental programme involving migration and division of the nucleus and the death of the parent conidium. An *atg8*Δ mutant of *M. grisea* that was impaired in autophagy prevented the death of the conidium and was non-pathogenic [54••,55••]. In *A. oryzae*, mutation of *ATG8* also prevented the formation of conidia and aerial hyphae [56].

In this context, deletion of *ATG9* in *S. cerevisiae* impairs sporulation [57,58]. However, deletion of *ATG9* in *C. albicans* had no effect on the yeast to hypha transition or the pathogenic interaction with host cells [59]. However, autophagy has been implicated in the induction of pseudohyphal growth in *S. cerevisiae* under conditions of nutrient stress [60] and is an important aspect of vacuole self-degradation and the destruction of other organelles of yeast [6]. Thus, vacuole biology and autophagy play a range of ubiquitous and organism-specific roles in fungal differentiation processes.

Vacuoles and fungal virulence

Mutations affecting vacuole function, biogenesis and inheritance often lead to defects in fungal virulence. In *C. albicans* several mutations affecting the vacuole have resulted in virulence defects in mouse models of systemic infection [43,46–48,61] or when challenged with macrophages *in vitro* [59]. In all cases, except for *MLT1*, these *C. albicans* mutants were defective in hypha formation [61]. A strain carrying a partially functional *VPS11* allele was not impaired in normal vacuole function, yet the vacuole was fragmented and defective in filamentation, but was not attenuated in a macrophage survival assay [62•]. A complete *vps11*Δ null mutant lacked a recognisable vacuolar compartment and was sensitive to stress, had reduced proteolytic activities, was severely defective in filamentation and was readily killed by macrophages [45]. These results suggest that vacuole expansion is required during germ tube emergence and for survival within the macrophage [62•].

In *U. maydis* the vacuolar transport chaperone Vtc4 was shown to be required for normal polyphosphate storage and a mutant strain in this gene was attenuated in

virulence on maize [63]. However, the deletion of Pho80, a component of the inorganic phosphate (P_i) acquisition in eukaryotic cells, did not lead to decreased virulence in *Aspergillus fumigatus* [64]. Although equivalent vacuole mutations have yet to be characterised in many species, it seems likely that a functional vacuole will prove to be essential for many aspects of the pathogenesis of a wide range of plant and human pathogenic fungi.

Conclusion

Recent studies demonstrate that vacuoles play central roles in fungal growth and differentiation as well as in well-established aspects of cellular physiology. Highly elongated, tubular vacuoles serve as conduits for nutrients and potentially for long-distance cell-to-cell signalling. Extensive vacuole expansion can substitute for cytoplasmic biosynthesis when nutrients are scarce, so that apical expansion and hence cell motility can occur with minimal biosynthetic demands. The vacuole volume occupies much of the total volume of a fungal cell and can therefore indirectly affect cell-cycle regulation and hence hyphal branching frequency. Finally, the turnover of key cellular proteins by autophagy has been implicated in appressorium formation and can act as a trigger that induces fungal differentiation. These features underline the vital contributions that vacuoles make to the growth and ecology of fungi.

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