Vesicular transport systems in fungi

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Canonical and unconventional mechanisms of secretion in many eukaryotic cells are relatively well known. In contrast to the situation in animal cells, mechanisms of secretion in fungi must include the capacity for trans-cell wall passage of macromolecules to the extracellular space. Although these mechanisms remain somewhat elusive, several studies in recent years have suggested that vesicular transport is required for trans-cell wall secretion of large molecules. Several fungal molecules, including proteins, lipids, polysaccharides and pigments, are released to the extracellular space in vesicles. In pathogenic fungi, a number of these vesicular components are associated with fungal virulence. Indeed, extracellular vesicles produced by fungi can interfere with the immunomodulatory activity of host cells. Fungal vesicles share many functional aspects with mammalian exosomes and extracellular vesicles produced by bacteria, plants and protozoa, but their cellular origin remains unknown. Here, we discuss the involvement of vesicular transport systems in fungal physiology and pathogenesis, making parallels with the mammalian, bacterial, protozoan and plant cell literature.

The capacity of fungi to produce copious amounts of extracellular molecules has been known for many decades. Pioneer studies on the ability of fungi to release enzymes to the extracellular space were carried out more than 50 years ago [1–3]. The highly efficient secretion of fungal hydrolytic enzymes, for example, account for the recycling of the most abundant polysaccharides in nature, cellulose and chitin. During the last four decades, many fungal extracellular proteins have been characterized and their secretion mechanisms have been elucidated. However, the pathways required for export of many other proteins, polysaccharides and pigments from the cytoplasm to the extracellular space remain largely unknown.

Protein export in eukaryotic cells has been studied in detail for the last three decades. Proteins that are destined to exit these cells can engage either conventional or unconventional secretory mechanisms [4,5]. In general, secreted proteins find their final destination at the plasma membrane level. In plant and fungal cells, however, secretion is a more complex event, because the plasma membrane is surrounded by a dense and complex molecular network, the cell wall (Figure 1) [6]. Therefore, a common characteristic to all secretion systems in fungi and plants is that extracellular molecules need to traverse the cell wall. This introduces a problem for molecules of large molecular mass since it has been difficult to envisage how these structures make their way through the dense fibrillar network that constitutes a cell wall.

Although eukaryotes were the most important prototypes for the seminal studies on secretory mechanisms, trans-cell wall secretion has been more deeply investigated in prokaryotes [7,8]. Bacteria have evolved complex and sophisticated trans-cell wall secretion systems that translocate cytoplasmic components to the extracellular space or even inject them into the cytoplasm of host cells [7]. In addition, Gram-negative bacteria ubiquitously release surface components to the extracellular milieu by forming outer membrane-derived extracellular vesicles (OMVs) [8]. OMVs are spherical structures of 20–250 nm in diameter that are produced when small portions of the outer membrane of Gram-negative bacteria bulge away from the cell. As recently reviewed by Kulp and Kuehn, soluble proteins are associated with OMVs as entrapped periplasm and as externally adherent material [8]. Importantly, OMVs can be released and diffuse far from the vesicle-producing cell, playing important roles in pathogenesis, quorum sensing, nutrition and gene transfer [8]. The role of OMVs as natural vehicles for the intercellular transport of virulence factors has been solidly established (for a review see [8]).

Extracellular vesicle release has recently been observed in Gram-positive bacteria and

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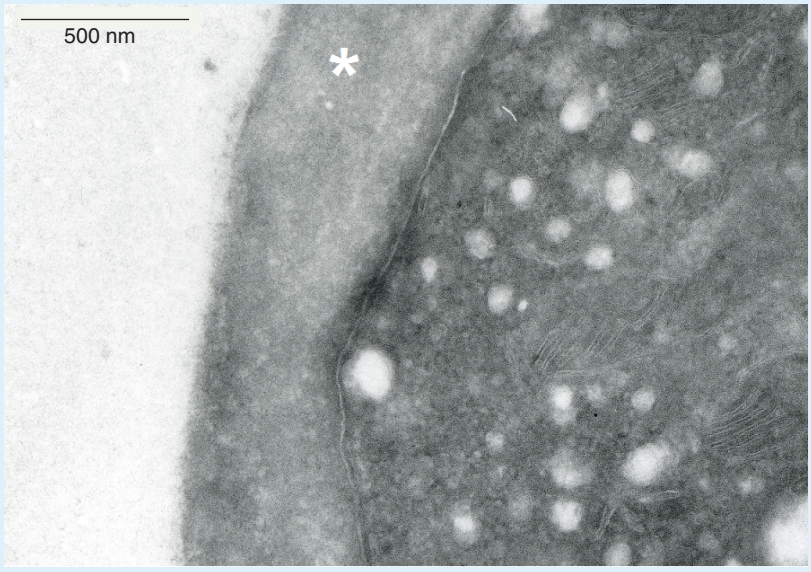

Figure 1. The fungal wall, illustrated in a *Cryptococcus neoformans* cell sectioned by cryo-ultramicrotomy and visualized by transmission electron microscopy. Fungal molecules destined to exit the cell must traverse this dense barrier to reach the extracellular space.
*: Fungal wall.
Image courtesy of Debora L Oliveira.

mycobacteria [9,10]. While OMVs derive from the external membrane of Gram-negative bacteria, the cellular nature of extracellular vesicles produced by Gram-positive species is still unknown. In contrast to Gram-negative bacteria, Gram-positive bacteria and mycobacteria have denser cell walls exterior to their plasma membranes, and thus have features more reminiscent of fungi and plants than the Gram-negative prototype. Like the situation for Gram-negative bacteria, extracellular vesicles released by Gram-positive pathogens are efficient carriers of toxins and virulence determinants [8–10]. For bacteria there is convincing evidence that extracellular vesicles are involved with pathogenesis. Some of the components that have been characterized in bacterial vesicles are potent immunogens that induce protective immunity, suggesting that they may also work in favor of the host during infection [10,11]. Bacterial extracellular vesicles, therefore, are promising vaccine candidates.

In contrast to what is observed for bacteria, trans-cell wall mechanisms in fungi and plants remain poorly understood. The lack of detailed knowledge regarding trans-cell wall secretion steps in fungi creates an interesting literature paradox, since extracellular enzyme production by yeast cells was first described more than 50 years ago [1–3]. In the last 5 years, a number of studies on the mechanisms evolved by fungal cells to promote trans-cell wall molecular transport have been published [12–26]. The current approaches developed for the study of fungal extracellular vesicles are summarized in Figure 2. The fungal pathogen *Cryptococcus neoformans* was the first model in which trans-cell wall transport was studied, as detailed later.

Production of extracellular vesicles by fungi: the *C. neoformans* model

The basidiomycete *C. neoformans* is a fungus that displays marked neurotropism during mammalian infection. Cryptococcal infections are characterized by the secretion of large amounts of polysaccharides to the extracellular space [27,28]. These polysaccharides will form its surface-associated capsule, which is essential for virulence [27]. Secreted cryptococcal polysaccharides include glucuronoxylomannan (GXM), the principal capsular component, and galactoxylomannan (GalXM), a minor constituent of the capsule network [27,28]. Although it is clear that polysaccharide secretion is very active in *C. neoformans*, knowledge on how these molecules reach the extracellular space is very preliminary.

More than a decade ago, the glycosphingolipid glucosylceramide (GlcCer) was characterized as a cell wall component of *C. neoformans* [29]. GlcCer is an important regulator of cryptococcal pathogenicity that is also targeted by antimicrobial antibodies [29–33]. Remarkably, this lipid appeared to be distributed into *C. neoformans* cell wall compartments resembling vesicular structures [29]. This observation led to the initial hypothesis that lipids could be transient cell wall constituents as part of a vesicular mechanism of trans-cell wall secretion. If true, this would imply the existence of extracellular vesicles containing GlcCer. Indeed, 7 years after the characterization of this lipid at the cryptococcal cell wall, our group isolated GlcCer-containing vesicles from *C. neoformans* culture supernatants [12]. These vesicles were abundantly detected not only in the cell wall microenvironment, but also in the capsular network of *C. neoformans* infecting the lungs of mice [12,29].

A number of studies suggested that the capsular polysaccharide GXM is synthesized in Golgi-derived compartments and then exported to the cell surface via vesicle-dependent mechanisms [34–38]. GXM is abundantly detected in different compartments of the cell wall and in the extracellular space [27,28], but mechanisms by which GXM traverses the cell wall remained obscure for many years. This problem is highlighted by the fact that GXM is a large polymer and it is

unclear how molecules of such high mass could diffuse across the tightly woven fibrillar network of the fungal cell wall. In *C. neoformans*, extracellular vesicle production and capsule enlargement were correlated [12]. Indeed, a number of different experimental approaches revealed that GXM is a constituent of vesicles that are released into culture supernatants of this fungus [12,18]. These observations, in combination with previous reports [34–36,38], led to the hypothesis that after synthesis in cytoplasmic compartments, GXM is loaded into secretory vesicles that traverse the cell wall to reach the extracellular milieu making the polysaccharide available to sustain capsule growth. This hypothesis was further supported by the observation that an acapsular mutant of *C. neoformans* efficiently used vesicular GXM for incorporation onto the cell surface [12] and by the suggestion that the minor capsular polysaccharide GalXM is also exported in vesicles [20]. These results confirmed that the fungus has mechanisms to obtain polysaccharides from extracellular vesicles and the predicted importance of vesicular secretion for capsule architecture.

The identification of a vesicular mechanism of polysaccharide trans-cell wall passage in the *C. neoformans* model generated important questions. Firstly, it was readily apparent that macromolecules other than polysaccharides could be exported to the extracellular space by a similar mechanism. Secondly, trans-cell wall vesicular

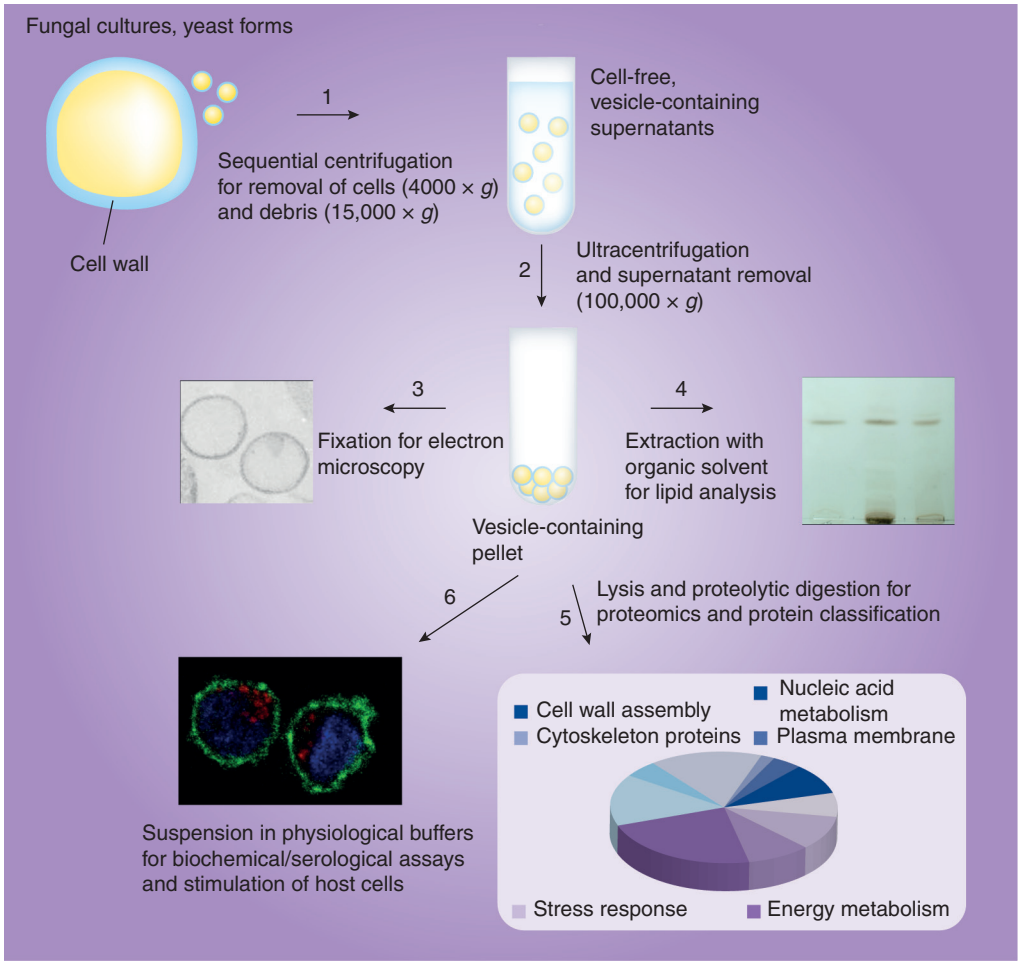

Figure 2. General steps required for isolation and multiple analyses of fungal extracellular vesicles. Cells and small debris are removed by centrifugation from fungal cultures (1) and supernatant vesicles are obtained by ultracentrifugation at 100,000 × *g* (2). These fractions are usually suitable for transmission electron microscopy (3), lipid analysis (4), proteomic characterization (5) and assays involving biochemical tests and/or stimulation of host cells (6). Illustrations in the steps described above represent transmission electron micrographs of *Cryptococcus neoformans* vesicle fractions (3), thin layer chromatography of vesicular extracts obtained from *Saccharomyces cerevisiae* (4), proteomic classification of *S. cerevisiae* vesicle proteins (5) and internalization of *C. neoformans* vesicles by murine macrophages (6). In (6), fungal vesicles are stained in red; macrophage plasma membranes appear in green and nuclei are stained in blue. For experimental details, see [12,16,22,23].

release could be a more general mechanism used by other fungi for macromolecular export. The morphological similarity between fungal vesicles and mammalian exosomes [13,15,16,39] also raised questions on their mechanisms of biogenesis. Thirdly, depending on their molecular composition, extracellular vesicles could be immunologically active, as well characterized for bacterial vesicles [10,40] and mammalian exosomes [41]. These questions were addressed in a number of studies that succeeded the initial description of extracellular vesicles in the *C. neoformans* model, as detailed in next sections.

Characterization of extracellular vesicle production in different species: molecular diversity in fungal vesicles

Albuquerque and colleagues also demonstrated that different ascomycetes produced extracellular vesicles [14]. In this study, extracellular vesicles produced by the dimorphic human pathogen *Histoplasma capsulatum* were characterized in detail, in association with the description of similar vesicles in *Candida albicans*, *Candida parapsilosis, Sporothrix schenckii* and *Saccharomyces cerevisiae* [14]. The proposal that extracellular vesicle release is a general mechanism in distantly related fungi was supported by a recent study by Vallejo and coworkers [25], who demonstrated the occurrence of extracellular vesicles in *Paracoccidiodes brasiliensis*. A recent study by Gehrmann and colleagues [42] and unpublished results from our laboratory suggest that the basidiomycetes *Malassezia sympodialis* and *Trichosporon asahii*, respectively, also produces extracellular vesicles [Joffe LS, Unpublished Data]. Although it is clear that the literature still requires confirmation of the production of extracellular vesicles by other species, this phenomenon has now been described for eight different fungal species. These reports support the notion that extracellular vesicle production is a general fungal phenomenon. As discussed later, the current literature supports the proposal that this mechanism would be an efficient strategy for fungal cells to release lipids, proteins, complex carbohydrates and pigments into the extracellular space.

For *H. capsulatum*, *C. neoformans* and *S. cerevisiae*, protein components of extracellular vesicles were characterized by proteomic approaches [13,14,23]. These analyses revealed a great compositional diversity, which included the characterization of a number of proteins of different cellular classes. In *S. cerevisiae*, protein composition of extracellular vesicles isolated from eight different strains grown under different temperatures (25 and 37°C) was very similar, supporting the consistency of the proteomic approach to determine vesicle components and a reduced susceptibility of protein composition to temperature shifts [23]. In *H. capsulatum* and *C. neoformans*, 206 and 76 vesicular proteins were identified, respectively [14,16]. Since the profiles of protein identification observed in these two species were similar to each other and also to that observed for *S. cerevisiae* [23], we speculate that discrepancy in individual proteins, as well as in the number of proteins identified, are due to differences in equipment and experimental approaches used for each species.

Several vesicular lipids were identified in extracellular vesicle fractions from *H. capsulatum, C. neoformans* and *S. cerevisiae* [13,14,23]. Sterols are apparently present in vesicles of all three species. Sterol identification was explored in greater detail in *C. neoformans*, resulting in the identification of ergosterol and an obtusifoliol-like structure as major components [12]. Phospholipids have been characterized in both *C. neoformans* and *H. capsulatum*. In the former, three different species of phosphatidylcholine were characterized by mass spectrometry [18]. In *H. capsulatum*, a lipidomic approach resulted in the identification of 18 different phospholipids, including species of phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine [14]. In addition to sterols and phospholipids, GlcCer, a glycosphingolipid, was characterized as a vesicular constituent in *C. neoformans* by serologic and spectrometric approaches [12]. Altogether, these results suggest that lipid composition of fungal extracellular vesicles is similar to that observed for the plasma membrane.

Complex carbohydrates have been also characterized in extracellular vesicles from different fungal pathogens. In *C. neoformans*, as mentioned earlier, a combination of transmission electron microscopy with serologic approaches, chromatographic separation and gradient centrifugation conclusively revealed that GXM is a p olysaccharide component of extracellularly released vesicles [12,18]. In the same model, analysis of monosaccharide composition in mutant cells that are not able to produce extracellular GXM suggested that GalXM is also exported in vesicles, which was supported by confocal microscopy using probes for detection of this polysaccharide [20]. In *P. brasiliensis*, extracellular vesicles contained α-galactosyl epitopes, which is in agreement with the export of fungal glycoproteins [25].

Morphologic analysis of approximately 400 individual vesicles released by *C. neoformans* into the extracellular space suggested that approximately 15% of this population had an electron dense content resembling pigments [16]. Although this observation represents indirect evidence for extracellular release of melanin, the fact that Einsenman and colleagues characterized vesicle-associated melanization in *C. neoformans* suggests that pigments can also be exported by vesicular mechanisms in fungi [19]. Particulate externalization of melanin has also been characterized in other fungi, such as recently shown for *C. albicans* [43].

Are extracellular vesicles involved in interaction of fungi with the host?

One remarkable property of extracellular vesicles of fungal pathogens is the fact that they carry molecules with functions suggesting that they biologically modify the pathogenic interaction with host cells [12–20,25]. In *C. neoformans*, 18 different components associated with fungal pathogenicity were identified by different approaches, including antioxidant proteins, GlcCer, GXM, heat shock proteins, laccase, urease, and enzymes required for capsule synthesis [12,13,16,18,19]. Antioxidant and heat shock proteins were also abundantly detected in *H. capsulatum* extracellular vesicles, as well as histones, which represent the targets of antimicrobial antibodies [14]. Several of the proteins found in extracellular vesicles produced by these two species were recognized by sera from infected patients [14,16], suggesting that these molecules are regularly secreted when *H. capsulatum* and *C. neoformans* are replicating within the host. In *P. brasiliensis*, vesicle components containing α-galactosyl epitopes reacted strongly with anti-α-Gal antibodies isolated from paracoccidioidomycosis patients [25]. These results strongly suggest that extracellular vesicle release may impact the interaction of fungi with their hosts.

Immunologically active extracellular vesicles have been consistently identified in bacteria, protozoan and mammalian cells [8–10,40,41,44–46]. In fact, immunization of mice with extracellular vesicles from diverse Gram-negative bacteria can induce protective responses in different pathogenic models [9–11]. In fungi, we recently demonstrated that vesicles released by *C. neoformans* to the extracellular space can modulate the functions of phagocytes [22]. In an *in vitro* model of phagocyte stimulation, apparently intact extracellular vesicles produced by *C. neoformans* were incorporated by mammalian macrophages. This phenomenon was associated with increased phagocytic indices and antimicrobial activity, and an augmented production of nitric oxide. Vesicle-stimulated macrophages also produced higher levels of TNF-α, IL-10 and TGF-β. In *Malassezia sympodialis*, release of extracellular vesicles inducing IL-4 and TNF-α has been described in patients with atopic eczema [42]. It remains unknown whether fungal extracellular vesicles are active during infection, but detection of released vesicles in both *in vivo* and *in vitro* models of infection supports this possibility [12].

The *S. cerevisiae* model: many questions on extracellular vesicle biogenesis

Secretory vesicles have been characterized in detail in *S. cerevisiae* over the last three decades ([47–50]; for review see [5]). The so-called canonical secretory pathway in yeast is governed by the *SEC* family of genes, which regulates protein traffic from the endoplasmic reticulum (ER) to the Golgi complex and from the Golgi complex to the cell surface [5]. Proteins that engage the conventional secretory pathway carry a secretion leader peptide, which provides a signal for the protein to traverse the ER and Golgi apparatus. However, many secreted proteins lack a signal peptide and exit the cell using the so-called 'unconventional' secretion mechanisms. As reviewed by Simons and Raposo, different unconventional secretion pathways may culminate with the release of vesicles to the extracellular space, which include exosomes, apoptotic blebs, microvesicles, microparticles, prostasomes and prominosomes [51].

The production of extracellular vesicles by eukaryotic cells originates from different cellular events. Exosomes are undoubtedly the most well-defined extracellular vesicles. Common characteristics for these structures include a diameter ranging between 40 and 100 nm and the ability to float on sucrose gradients to a density that ranges from 1.13 to 1.19 g/ml (for review see [51]). The biogenesis of exosomes is thought to occur within endosome-derived multivesicular bodies (MVBs). Exosomes then correspond to the intraluminal vesicles of endosomal MVBs that fuse with the cell surface in an exocytic manner, resulting in the release of vesicles to the extracellular space. Therefore, exosomes are enriched in proteins that are found in MVB intraluminal vesicles [51]. Formation of MVB-like structures in fungi has been well documented [16,52–54]. In some of these reports, MVBs appeared to undergo fusion with the plasma membrane, resulting in the release of vesicles to the

periplasmic space [16,52,53]. In fact, extracellular vesicles produced by *C. neoformans* were recently defined as fungal exosomes [26], but three different proteomic studies revealed that the majority of proteins identified in these vesicles differ from those found in mammalian exosomes [14,16,23,51,55]. Fungal vesicles also showed average diameters that were higher than those found for mammalian exosomes [12,16,19]. Altogether, these observations suggest that fungal extracellular vesicles share compositional and morphological aspects with mammalian exosomes, but they also diverge in key characteristics. This scenario would be in agreement with the supposition that fungal extracellular vesicles could have different, or at least additional, pathways of biogenesis.

A systematic study of vesicle characterization in a number of secretion mutants of *S. cerevisiae* supported the hypothesis that additional biogenesis pathways exist [21,23]. Although extracellular vesicles produced by yeast mutants with defective formation of MVBs had an altered protein composition, wild-type (WT) and mutant cells were similarly efficient in producing extracellular vesicles [23]. This observation argues strongly against the hypothesis that extracellular vesicles produced by fungi correspond exactly to exosomes. There is also concrete evidence that Golgi-derived secretion pathways seem to deeply affect formation of fungal extracellular vesicles. First, interference with *SEC6* (a component of the post-Golgi conventional secretory pathway) expression in *C. neoformans* has been reported to abolish extracellular vesicle formation [26]. Second, a quantitative comparison of vesicle formation in *S. cerevisiae* WT cells and in mutant cells defective in the expression of *SEC4* (another component of the post-Golgi conventional secretory pathway) revealed that the mutant was significantly less efficient at producing extracellular vesicles than the parental strain [23]. Finally, inhibition of post-Golgi secretion pathways with brefeldin A affected the export of vesicular components to the extracellular space in *C. neoformans* [37].

The requirement of Golgi-associated components for extracellular vesicle formation is controversial and still very obscure. Post-Golgi secretory vesicles are expected to fuse with the plasma membrane, releasing their components extracellularly and disappearing after fusion events. It is unclear whether the conventional post-Golgi secretion pathway in fungi is branched such that it could be possibly implicated in extracellular vesicle release. It is noteworthy that there is no evidence in the literature supporting this hypothesis. However, it seems unquestionable that post-Golgi secretory steps are required for extracellular vesicle production in fungi, which poses additional and unknown mechanisms related to the coordination of unconventional secretion in fungal cells. The hypotheses raised earlier are summarized in Figure 3.

Unconventional polysaccharide secretion in fungi: the role of the Golgi reassembly and stacking protein

In *S. cerevisiae*, it has been suggested that mutant cells lacking Grh1, the yeast homolog of mammalian Golgi reassembly and stacking protein (GRASP), are less efficient than WT cells in producing extracellular vesicles [23]. GRASP has been shown to regulate unconventional extracellular delivery of cytoplasmic proteins that lack a signal peptide and the transport of transmembrane proteins to the plasma membrane, sidestepping conventional mechanisms of secretion [56,57]. More precisely, it has been demonstrated in *Dictyostelium discoides* that GRASP regulates an unconventional mechanism of secretion of acyl-CoA-binding protein that bypasses the usual route for Golgi-dependent membrane traffic [58].

In the study mentioned earlier [58], it was speculated that the unconventional secretion of acyl-CoA-binding protein could involve vesicular or nonvesicular routes, but not ABC transporters. At that time, it was suggested that acyl-CoA-binding protein would be transported from the cytosol into the internal vesicles of MVBs, which would then fuse with the plasma membrane to release the internal vesicles as exosomes [59]. Further studies on the role of GRASP in fungal cells demonstrated that GRASP-dependent unconventional secretion in *S. cerevisiae* and *Pichia pastoris* required autophagy proteins [60,61]. According to these models, acyl-coenzyme A-binding protein is sequestered into autophagic vesicles that are oriented to the plasma membrane where their content is released into the extracellular space [60–62]. This alternative mechanism, the so-called exophagy, would likely result in the release of vesicles to the extracellular space [62]. Both hypotheses – exosome release and exophagy – would be in agreement with the observation that yeast cells lacking GRASP homologs are less efficient than WT cells at producing extracellular vesicles [23].

In the *C. neoformans* model, vesicular transport of polysaccharide molecules to the cell surface was affected by mutations in genes (*SAV1* and *SEC6*) that were demonstrated to be required

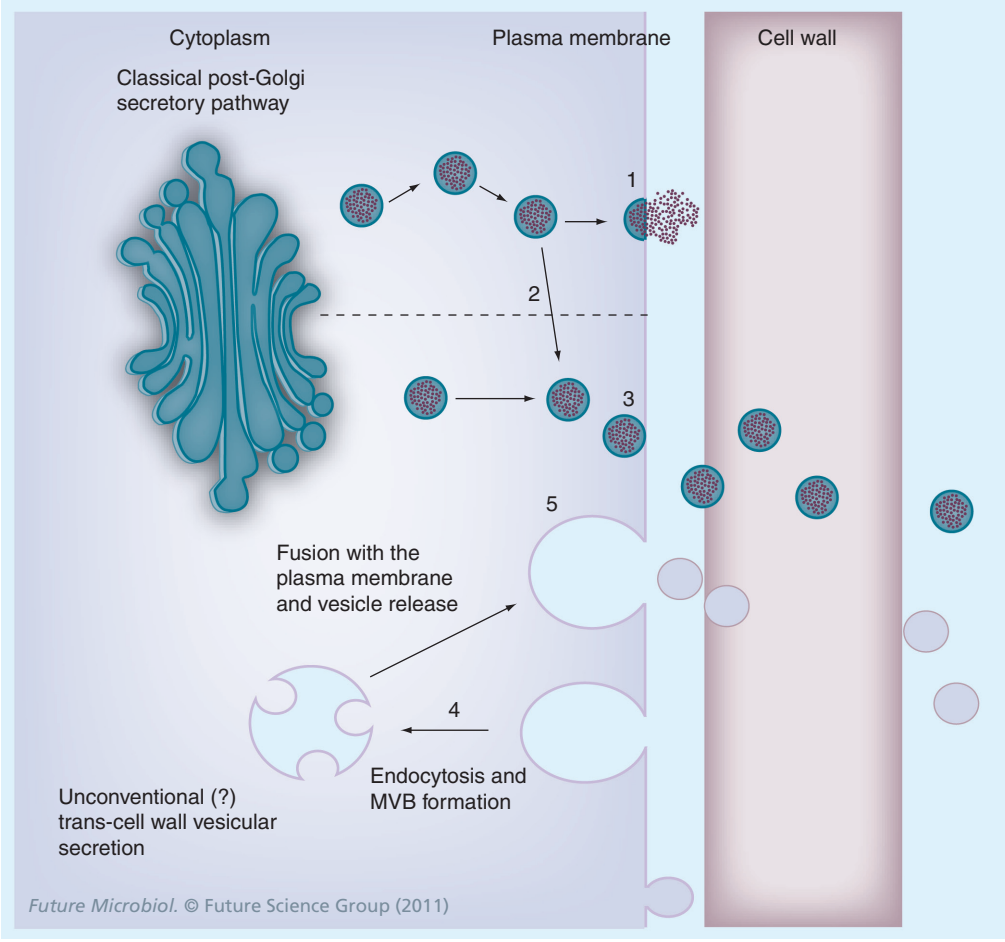

Figure 3. Secretion pathways potentially required for extracellular vesicle release by fungal cells. In eukaryotes, post-Golgi, conventional pathways for protein secretion result in fusion of secretory vesicles with the plasma membrane and release of vesicular content into the extracellular space (1). The characterization of *Saccharomyces cerevisiae* Sec4p, a member of the conventional secretory network, as a regulator of production and or release of fungal extracellular vesicles [23] suggests that both conventional and unconventional secretory pathways may interact (2), although the literature lacks experimental evidence to fully support this hypothesis at present. Additional studies indicate that members of the conventional secretory pathway regulate extracellular vesicle release (3) through still unknown mechanisms [23,26]. Similarities in morphology and composition suggest that fungal extracellular vesicles are related to mammalian exosomes, which originate from endocytic events resulting in MVB formation (4). MVBs can fuse with the plasma membrane resulting in extracellular vesicle release (5). For extracellular vesicle formation, we cannot rule out the requirement of still poorly characterized unconventional secretory pathways (e.g., the Golgi reassembly and stacking protein-mediated secretion [66] and exophagy [62]), as well as membrane budding (6). It remains unknown how the vesicles traverse the cell wall to reach the extracellular space. The term 'unconventional' is labeled with a question mark because it is still unknown if both concepts and nomenclature used for protein secretion would be applicable to extracellular vesicle release in fungi. Differential tones of extracellular vesicles in this scheme represent the possibility of variable composition depending on cellular origin.
MVB: Multivesicular body.

for conventional mechanisms of exocytosis in other models [26,34]. However, a remarkable observation was that capsule assembly appeared normal in those mutants. This observation led to the hypothesis that polysaccharide secretion could also involve elements of unconventional secretory pathways. The observation that extracellular vesicles produced by *C. neoformans* are loaded with capsular polysaccharides [12,18] and the apparent link between GRASP-dependent unconventional secretion and formation of extracellular vesicles in yeast [58–61,63] were the basis for the analysis of the role of GRASP homologs in *C. neoformans* polysaccharide export.

C. neoformans has a single *GRASP* ortholog that shows the typical PDZ-like domains in

the N-terminal region of GRASP [24]. Deletion of the *C. neoformans* GRASP ortholog did not affect classic virulence factors, such as growth at 37°C, extracellular urease activity and melanin formation. Mutant cells lacking the GRASP ortholog, on the other hand, showed an impaired ability to secrete GXM, produced smaller capsules and shorter capsular polysaccharide molecules, in comparison with WT and reconstituted cells [24]. These alterations coincided with reduced virulence and increased susceptibility of the mutant to the antimicrobial activity of macrophages [24].

The mechanisms by which GRASP regulates polysaccharide secretion are still unknown. Lack of GRASP could affect export of GXM-containing extracellular vesicles to the extracellular space, which would be in agreement with the roles suggested for this protein in other models [58–63]. However, recent results from our group suggest that *C. neoformans* cells lacking GRASP showed normal release of extracellular vesicles [64]. This information contrasts with the fact that a *S. cerevisiae grh1* mutant manifests a reduced ability to produce extracellular vesicles [23] suggesting that *C. neoformans* GRASP could be alternatively required for loading GXM into secretory vesicles. This hypothesis is supported by the fact that vesicles produced by a *C. neoformans grasp* mutant contain smaller amounts of GXM [64]. Finally, lack of GRASP could affect important cellular sites of the secretory machinery. In fact, it has been suggested that in *S. cerevisiae* and *P. pastoris* the GRASP homolog is functional at the trans-ER–Golgi interface [65]. This observation would be in agreement with the fact that brefeldin A, which inhibits transport of proteins from the ER to Golgi, also strongly inhibits GXM secretion and capsule assembly in *C. neoformans* [37].

Vesicular production in fungi: what's next?

Vesicle biogenesis

Knowledge on the properties of fungal extracellular vesicles has unquestionably advanced in the last 5 years, but numerous questions remain open. While vesicle composition is fairly well characterized for the major components, it is likely that most minor components remain to be identified. Perhaps most enigmatic are the mechanisms for fungal vesicle biogenesis. Studies involving point mutations in genes of secretory pathways have thus far proved unsuccessful in shutting down extracellular vesicle production. This observation may reflect the hypothesis that multiple mechanisms are required for production and/or release of fungal extracellular vesicles and/or that the process is highly redundant, as supported by the fact that the methods currently used for preparation of extracellular vesicle fractions may coisolate vesicular compartments from different cellular origins. The possibility that there are still several unknown pathways for the formation and release of extracellular vesicles in fungi cannot be ruled out. In this regard, studies involving multiple mutations in secretory pathways in combination with the use of up-to-date techniques of cell biology and microscopy may be clarifying. Most models explored so far are based on mutants where protein secretion is impaired. Considering that vesicle formation is dependent on intense membrane rearrangement, evaluating the role of lipids in these processes is likely to be promising.

What is the physiological function of fungal extracellular vesicles?

The complex composition of extracellular vesicle fractions obtained from fungal supernatants suggests that vesicle formation may include random incorporation of cytoplasmic and surface components. This observation would agree with the hypothesis that extracellular vesicle formation is required for cellular recycling of surface/cytoplasmic components. On the other hand, extracellular vesicle fractions also include components that follow, in other cellular systems, well-defined secretory mechanisms, as exemplified by melanin. Therefore, the need for probes to track cellular pathways related to extracellular vesicle formation seems clear.

What is the impact of vesicle formation during fungal infections?

Although two independent studies have unequivocally proven that fungal vesicles are immunologically active [22,42], it remains unknown whether they are produced during human or animal infections. If this is the case and fungal cells release vesicles containing pathogenic determinants *in vivo*, it would be necessary to learn whether extracellular vesicles would reach concentration ranges at which they would effectively affect the physiology of host cells and tissues. Such analyses would necessarily include assays aiming at the determination of how stable fungal vesicles are, as well as when they are optimally produced during the fungal cell cycle. This is an interesting, but arduous, line of experimentation rendering probably one of the most complex issues in the understanding of the biological functions of fungal extracellular vesicles.

Polysaccharide export: canonical or unconventional mechanisms of secretion?

Cellular pathways required for protein secretion also appear to be functional for polysaccharide export in the *C. neoformans* model. However, blocking either conventional or unconventional mechanisms of secretion in this fungus only generates partial or very subtle phenotypes. One possibility is that *C. neoformans* uses different, but complementary, mechanisms for polysaccharide export, which could be targeted to different extracellular locations. The finding that mutants with defects in conventional secretion (*SEC4* and *SEC6* genes) accumulate cytoplasmic GXM-containing cytoplasmic vesicles [26,34] is consistent with a role for this pathway in polysaccharide export. However, these mutants have apparently normal capsules [26,34]. On the other hand, the *grasp* mutant of *C. neoformans* manifests a clear defect in capsule formation in association with a decreased concentration of extracellular GXM, but mutant cells are still encapsulated [24]. These observations may suggest that GXM export in *C. neoformans* is a redundant cellular event that may involve convergent biosynthetic pathways.

Conclusion

From the first description of trans-cell wall vesicular transport in *C. neoformans* in 2007 [12], our knowledge on how fungal cells use vesicular transport systems to export extracellular molecules has rapidly grown. The advanced knowledge on composition, morphology, immunological activity and biochemistry of extracellular vesicles, however, engendered many new questions to how vesicle formation and release are regulated. Fungal vesicles also appear to be efficient vehicles for polysaccharide secretion, but we do not know if the pathways used for both conventional and unconventional protein secretion are the same as those required for polysaccharide traffic. In addition, recent studies have indicated that unconventional pathways of protein secretion are more numerous than previously thought [4], suggesting that polysaccharide release may also require multiple and still unknown cellular mechanisms of secretion. As recently discussed by our group [21], trans-cell wall secretion in fungal cells is a cell biology topic that still comprises many more questions than answers. However, it is clear that the new scenario posed by the discovery of fungal extracellular vesicles has revealed complex and still poorly characterized events with the potential to impact both cellular physiology and pathogenesis in fungi.

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Executive summary

- Mechanisms by which extracellular fungal macromolecules traverse the cell wall have remained unknown for five decades.
- The recent discovery of extracellular vesicles in the basidiomycete pathogen *Cryptococcus neoformans* and other ascomycetes suggest that fungal cells use vesicular systems of trans-cell wall transport to release molecules of different natures to the extracellular space.
- Mechanisms of vesicular transport appear to be closely linked to polysaccharide traffic in fungal cells.
- These interesting yet still obscure events are potentially relevant for both the physiology and pathogenesis of fungal cells, but the cellular origin of extracellular vesicles in fungi remains elusive.
- In this article we discuss what is known about vesicular transport systems in fungi and the numerous questions originating from these recent findings.

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- ▪ of interest
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