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Deciphering Fungal Extracellular Vesicles: From Cell Biology to Pathogenesis

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Abstract

Purpose of Review Extracellular vesicles (EVs) have been implicated in the pathogenesis of several infectious diseases, but their role in fungal virulence has only recently started to emerge. Here we summarize recent discoveries in this field and highlight areas where more research is urgently needed.

Recent Findings In fungi, EVs carry a heterogenous molecular content including polysaccharides, lipids, allergens, heat-shock proteins (HSPs), enzymes, pigments, and RNAs. Many of these components have been shown to modulate the host immune system and to participate in biofilm production, drug resistance, cell communication, and virulence enhacement. However, the influence of EVs on the course of fungal disease remains elusive, primarily due to technical limitations. Nevertheless, there are many potential medical applications yet to be investigated, including the development of diagnostic tools, vaccine adjuvants, and design of antifungal agents.

Summary Although at a very early stage, the field of fungal EVs is rapidly expanding and holds exciting potential to strongly impact on our understanding and treatment of this group of diseases.

Keywords Fungi · Immune modulation · Secretion · Extracellular vesicles · Virulence and yeast

Introduction

The release of extracellular vesicles (EVs) is a conserved phenomenon among all forms of life [1]. EV play important roles in different diseases, including cancer metastasis [2], inflammatory disorders [3], prion horizontal transmission [4], spread of bacterial antibiotic resistance and toxin release [5], as well as exchange of genetic material between host cells and protozoans [6]. In fungi, EV structures are composed of bilayered membranes and a heterogeneous cargo that includes lipids, polysacharides, proteins, and nucleic acids. Many of these components are virulence-related molecules that are delivered to the extracellular space [7•, 8•]. Recently, EVs have been implicated in intercellular communication processes that involve the transfer of virulence traits from a pathogenic yeast to an environmental strain [9••].

Fungal EVs were first described in the supernatant cultures of the yeast pathogen *Cryptococcus neoformans* [10•]. Subsequently, EVs were reported in other fungal species of medical and/or biotechnology importance, including *Histoplasma capsulatum* [11], *Candida albicans* [11, 12], *Saccharomyces cerevisiae* [11, 13], *Malassezia sympodialis* [14, 15], *Paracoccidioides brasiliensis* [16], *Alternaria infectoria* [17], *Pichia fermentans* [18], *Sporothrix schenckii* [11], *S. brasiliensis* [19], *C. gattii* [9••], and *Trichophyton interdigitale* [20].

Several different mechanisms are used by fungi to produce EVs. Some EVs are generated from the conventional secretory pathway with the participation of the endoplasmic reticulum, involving the fusion of vesicles derived from the Golgi apparatus with the plasma membrane [21]. Alternatively, via the unconventional pathway, EV formation occurs through

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plasma membrane invagination, creating endosomes that may develop internal vesicles and result in multivesicular bodies (MVBs) [22]. Although MVBs generally fuse with lysosomes, they may also merge with the plasma membrane, delivering their internal content (exosomes) into the extracellular space [12, 23]. EVs can also be formed from the evagination of the plasma membrane (inverted macropinocytosis), followed by release of microvesicles, also known as ectosomes [23, 24]. For consistency, all vesicle populations will be refered as vesicles or EVs in this review.

EVs originating from fungal cells need to cross the cell wall in order to reach the extracellular environment. There are three hypotheses to explain how EVs overcome this physical barrier: (i) by protein channels formed by the rearrangement of the cytoskeleton; (ii) with the participation of enzymes that would temporarily remodel the cell wall; or (iii) forcing their passage through pores [23, 25]. Most recently, images of intact liposomes reaching the fungal membrane demonstrated the dynamic properties of the fungal cell wall [26]. Therefore, it has been speculated that this mechanism could be reversed to deliver vesicles from the membrane to the outer cell wall. Furthermore, the size of pores in fungal cell wall could enlarge up to 400 nm, aiming to increase nutrient uptake as a response to environmental stress [27].

EVs become unstable and lyse almost immediately in the presence of serum proteins [8•]. Albumin might mediate EV rupture by binding to ergosterol, destabilizing vesicle membranes. This finding suggests that, in the course of infection, EV integrity is short-lived following their release from fungal cells [8•]. Recently, it was shown that mammalian β-galactoside-binding protein galectin-3 (Gal-3) produced a direct lytic effect on *C. neoformans* EVs [28]. This lectin presents an extensive distribution on the surface of host cells as well as intracellularly [29], and has influenced host response against different fungal infections [30–32]. For instance, Gal-3 levels were increased in tissues and serum of mice infected with *C. neoformans* and contributed to a higher killing rate in Gal-3-deficient mice [28].

The participation of EVs on fungal biology and pathogenesis remains elusive [33, 34]. However, previous studies have correlated fungal EVs with protein metabolism [35], morphological differentiation [18], immune modulation of the host [36•], participation in cell communication [9••], and fungal dissemination [37•]. In this context, this review will focus on findings related to EVs from the main fungal human pathogens, the techinical limitations that currently prevent the development of this field, and its ultimately promising clinical applications.

Cryptococcus spp.

Cryptococcus is the fungal model in which fungal EVs have been most intensively studied to date. The first description of fungal EVs was in fractionated culture supernatants of *C. neoformans* [10•]. These vesicular compartments were identified as spherical bilayered membranes enriched in lipids including glucosylceramide, ergosterol, and sterol. The presence of virulence-related molecules, including the major capsular polysaccharide glucuronoxylomannan (GXM), was the basis for the classification of these compartments as *virulence bags* [7•]. GXM molecules are synthesized intracellularly, transported within vesicles from the Golgi complex, and secreted via exocytosis [38]. The Golgi reassembly and stacking protein (GRASP) may be involved in the process of loading GXM into vesicles and fusion with the plasma membrane as well as with RNA export [39, 40]. A mutant strain lacking *GRASP* in *C. neoformans* showed alterations in Golgi morphology, hypocapsular phenotype, and reduced GXM secretion. In a mouse model, *grasp*Δ cells were easily phagocytosed and presented attenued virulence, causing late lethality in infected animals. In contrast, levels of urease activity and melanin biosynthesis were not affected in these cells [39].

Melanin production increases *C. neoformans* resistance towards host-derived reactive oxygen molecules [41]. EVs from *C. neoformans* were able to melanize after incubation with L-3,4-dihydroxyphenylalanine (L-DOPA) [42], suggesting that partitioning of melanin within these compartments may allow *Cryptococcus* cells avoid the toxic effects of the intermediates generated by oxidation of L-DOPA [42].

Proteomic analysis of *C. neoformans* vesicles identified proteins related to protection against oxidative stress, including HSPs, superoxide dismutase, thiol-specific antioxidants, and catalases [7•]. However, EV cargoes are complex and not restricted to virulence factors. Molecules related to cell biology were also detected in EVs, including proteins associated with carbohydrate metabolism, cell organization, molecular transporters, protein degradation, histones, and ribosomal proteins [7•, 13].

EVs present immunological properties (Table 1). The stimulation of macrophages by *C. neoformans* EVs increased the levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-10, transforming growth factor-beta (TGF-β), and nitric oxide, enhancing phagocytosis index and macrophage antimicrobial activity [36•]. EVs released by *C. neoformans* were also progressively internalized into the cytoplasm of human brain microvascular endothelial cells, leading to increased yeast transmigration through the blood-brain barrier. This process relied on the redistribution of β-actin, caveolin-1, and vimentin, which induced fusion with host cells [37•]. As with mammalian host cells, the soil amoeba *Acanthamoeba castellanii*, which is an environmental predator of *Cryptococcus* cells, is able to internalize *C. neoformans* EVs and this internalization subsequently enhances yeast survival (Fig. 1) [44].

Genes involved on secretion mechanisms are evolutionary conserved among fungal species. In yeast, the protein Sec6 participates in EV fusion with the plasma membrane [46] and

Table 1 Immunogenic propreties of fungal extracelular vesicles

Fungal pathogens	Host cells	Immunogenic properties	References
Candida albicans	Macrophages	↑ NO, IL-12, TGF-β, IL-10, ↑ CD86	[43••]
	DCs	↑ IL-10, IL-12p40, TNF-α ↑ CD86 and MHC-II	[43••]
Cryptococcus neoformans	Macrophages	↑ TNF-α, IL-10, TGF-β, NO ↑ Phagocytosis and fungicidal activity	[36•]
	Brain endotelial cells	↑ Transmigration	[37•]
	Acanthamoeba castellanii	↑ Yeast survival	[44]
C. gattii	Macrophages	↑ Intracellular proliferation	[9••]
Malassezia sympodialis	Peripheral blood mononuclear cells	↑ IL-4	[14]
Paracoccidioides brasiliensis	Macrophages	↑ TNF-α, IL-6, IL-12 M1 polarization ↑ Fungicidal activity	[45]
	DCs	EV recognition by C-type lectin receptors DC-SIGN and DC-SIGNR	[45]
Sporothrix brasiliensis	DCs	↑ Phagocytosis ↑ IL-12p40, TNF-α, and IFN-	[19]
Trichophyton interdigitale	BMDMs	↑ TNF-α, IL-6, and iNOS, with participation of TLR2 M1 polarization	[20]
	Keratinocytes	↑ NO, TNF-α, IL-6, IL-1-β, IL-8	[20]

NO nitric oxide; *IL-* interleukin; *TGF-β* transforming growth factor-beta; *CD86* cluster of differentiation 86; *DCs* dendritic cells; *TNF-α* tumor necrosis factor-alpha; *MHC-II* major histocompatibility complex II; *DC-SIGN* dendritic cell-specific intercelular adhesion molecule-3-grabbing non-integrin; *DC-SIGNR* dendritic cell-specific intercelular adhesion molecule-3-grabbing non-integrin-related protein; *IFN-γ* interferon-gamma; *BMDMs* bone marrow-derived macrophages; *TLR-2* toll-like receptor-2

reduction of Sec6 expression by RNA interference leads to intracellular accumulation of vesicles and a lack of laccase or urease secretion. Mutants lacking the *SAV1* gene in *C. neoformans* do not secrete acid phosphatase and accumulate cytoplasmic vesicles containing GXM [38]. In contrast, a Sec14 homologue is essential for the secretion of phospholipase B1 (Plb1) but not for the secretion of GXM or laccase [21, 47]. Modulating traditional secretory genes such as *SEC1*, *SEC4*, and *SEC6*, as well as the vacuolar protein sorting (VPS), affects EV composition and their release

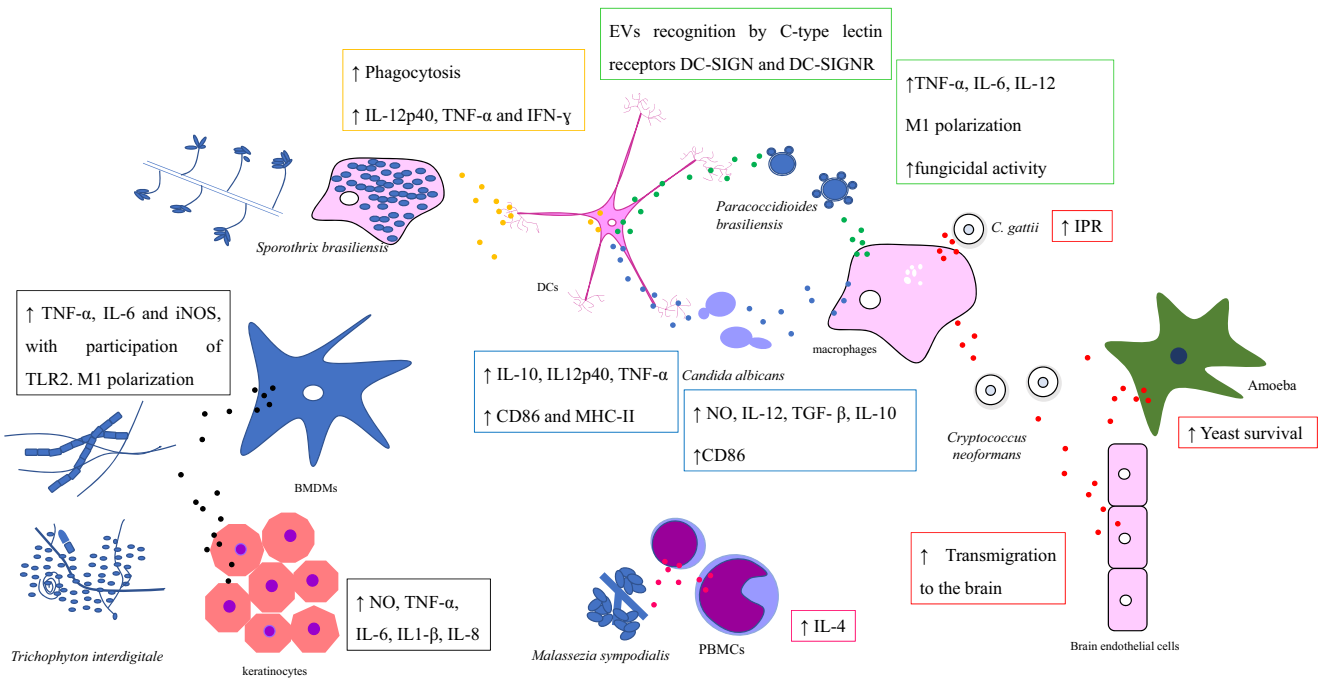

Fig. 1 Impact of fungal extracellular vesicles (EVs) in the host cells. EVs: *Sporothrix brasiliensis* (yellow), *Trychophyton interdigitale* (black), *Paracoccidioides brasiliensis* (green), *Candida albicans* (blue), *Malassezia sympodialis* (pink), *Cryptococcus neoformans* and *C. gattii* (red)

kinetics [13, 48, 49]—an effect which, in some cases, leads to an impact on virulence [50, 51]. Genes and molecules that influence the fungal secretory system are summarized in Table 2.

Proteins involved in lipid distribution on the fungal plasma membrane, such as the aminophospholipid translocase 1 (Apt1) and scramblases [52, 53, 59••], have also an important impact on membrane fusion events and therefore fungal secretion. Mutant cells lacking Apt1 produced EVs of varying sizes and reduced GXM content when compared to wild-type strain. Apt1 mutants show decreased macrophage infection and organ colonization, but whether that is directly associated with an altered EV profile remains unknown [52, 53]. On the other hand, a mutant lacking scramblase in *C. gattii* produced vesicles with unaltered GXM amounts, although EVs from the mutant had larger size and altered RNA profiles [59••].

Interestingly, the absence of any of these proteins was associated with complete elimination of EV release [13, 49], presumably reflecting a requirement for a "wild-type" membrane composition to generate EVs [22–24]. In contrast, higher release of EVs was observed in the absence of a putative G1/S cyclin in *C. neoformans*. However, rather than driving increased virulence, this phenotype in fact reduced virulence in murine macrophages and *G. mellonella* infection [55].

Candida spp.

Transmission electron microscopy of vesicles from *Candida albicans* and *C. parapsilosis* were described by Albuquerque et al. [11]. Overall, the structural morphology of the vesicles were similar to other fungal EVs [10•, 11]. As in *C. neoformans*, vesicles from *C. albicans* also can be internalized by host immune cells, for instance macrophages and dendritic cells (DCs) leading to increased cytokine production [43••] (Fig. 1).

Major components of both *C. neoformans* and *C. albicans* EVs are miRNA-like and iRNA sequences, which has led to the suggestion that these molecules could play a role during fungal infection by mimicking endogenous miRNA and regulating gene expression in host cells [60••]. The converse situation, in which host cells secrete vesicles containing small RNAs to silence pathogenesis-related genes in pathogens, has already been documented for *Arabidopsis thaliana* and the fungal pathogen *Botrytis cinerea* [61], suggesting that such a mechanism could theoretically occur.

As with *C. neoformans*, phospholipid biosynthesis in *C. albicans* affected EV morphology, composition, and immunogenicity. Mutants lacking the gene for phosphatidylserine synthase (*CHO1*) or for phosphatidylserine decarboxylase (*PSD1* and *PSD2*) produced EVs with increased PC and significantly altered protein content. Interestingly, EVs from

Table 2 Genes and molecules related to fungal secretion pathways

Genes/molecules	Relation to secretion/virulence	References
Albumin	EV disruption	[8•]
APT1	EVs with different sizes and reduced GXM content. Diminished survival in macrophages. Attenuated virulence in mice	[52, 53]
CHO1	EVs unable to activate NF-ƙB	[54]
CLN1	Increased release of EVs. Lower intracelular proliferation and attenuated virulence in vivo	[55]
EDTA	Decrease in EVs, free-GXM, and biofilm production	[56]
ESCRT	Decreased EV release and fluconazole biofilm resistance	[57••]
Gal-3	EV disruption	[28]
GRASP	GXM load into EVs. Affected EV sizes and RNA content	[40, 58]
LAC1 and *LAC2*	Incorporation of melanin in the cell wall by an enzyme-independent mechanism	[42]
miR210 and miR-26	Differentiation from yeast-like to pseudohyphal form	[18]
PSD1 and *PSD2*	Larger EVs	[54]
SAV1/SEC4	GXM synthesis and transport. Secretion of phosphatase acid	[21, 38]
AIM25	Larger EVs	[59••]
SEC1	EV fusion with the plasma membrane	[21, 38, 48, 49]
SEC14	Lipid metabolism and secretion of phospholipase B	[21]
SEC6	EV fusion with the plasma membrane. Affected secretion of laccase, urease, and GXM	[21, 48]
SNF7	Involved in multivesicular body formation. Reduced secretion of GXM, melanin, and capsule size. Attenuated virulence in mice	[51]
VPS23	Capsule deficiency and attenuated virulence in experimental model	[50]
VPS34	Reduced melanin formation	[52]

EVs extracellular vesicles, *GXM* glucuronoxylomannan, *NF*-ƙB factor nuclear kappa B, *EDTA* ethylenediamine tetraacetic acid, *Gal-3* galectin-3

psd1Δpsd2Δ were larger, whereas *cho1Δ* EVs were unable to activate NF-ƙB in bone marrow-derived macrophages (BMDMs) [54].

A comparative study between the proteome of *C. albicans* EVs and EV-free secretions showed that most proteins in the latter were secreted by the classical pathway, including hydrolases, adhesins, and proteins involved in biofilm formation [62]. This latter finding aligns with recent data showing that *Candida* biofilms release EVs with a different content from planktonic cells [57••].

Other Fungal Species

Studies describing EVs from filamentous fungi are rare [34]. *Alternaria infectoria* was the first filamentous fungus to be described secreting EVs [17]. Analysis of their content identified 20 proteins, seven of which had previously been reported in other fungal vesicles. However, a higher number of proteins related to DNA repair and replication were observed. Although *A. infectoria* contains melanin on their cell wall, laccase was not found. Nevertheless, the enzyme polyketide synthase was detected, which catalyzes a melanin intermediate and is associated with pathogenesis in various fungi, including the melanin producer *C. neoformans* [17, 42]. Molecules related to cell adhesion, transport, metabolism, and plasma membrane were also found. Interestingly, the authors detected Hsp60, which was also found in EVs from the fatal human pathogen *Histoplasma capsulatum* [11, 17]. In addition to numerous other proteins involved in pathogenesis and host immune response [11, 63], *Histoplasma* EVs also show interesting strain-specific differences in their RNA content, the impact of which remains unknown [63].

EVs have also been purified and partially characterized from *Saccharomyces cerevisiae*, *Sporothrix schenckii* and *S. brasiliensis* [11, 19] as well as the human skin commensal yeast, *Malassezia sympodialis* [14]. As in other fungal species, small RNAs were identified within *M. sympodialis* vesicles [64]. Since EVs from *M. sympodialis* are actively internalized by keratinocytes and monocytes (Fig. 1), it has been suggested that these EVs could participate during the skin inflammation process by delivering allergens to the host cells in atopic eczema patients [15].

Vesicles have also been identified from the dermatophyte *Trichophyton interdigitale*, another skin fungal pathogen, and shown to induce a dose-dependent proinflammatory response in keratinocytes and BMDMs [20]. Similarly in the thermodimorphic fungus *Paracoccidioides brasiliensis*, EVs promoted the release of proinflammatory cytokines, such as TNF-α, IL-6, and IL-12, and favored M1-polarization (Fig. 1) [45]. Previously, *P. brasiliensis* EVs were reported to carry α-linked galactosyl epitopes—highly immunogenic molecules that are likely to be stored inside vacuoles resembling multivesicular bodies [65]. Proteomic [66], lipidomic [16], and glycobiome [67] analysis of *P. brasiliensis* EVs have been performed. One interesting outcome of these studies was the finding that oligosaccharides exposed on EVs surface are mainly composed of high amounts of mannose and lower amounts of *N*-acetylglucosamine and can be recognized by the mammalian C-type lectin receptors DC-SIGN and DC-SIGNR [67] (Fig. 1).

Limitations and Challenges

Despite the considerable progress made in recent years, the fungal EV field suffers from a number of significant technical limitations at present. Foremost among these is that methods for isolation and quantification of the fungal vesicles are laborious and inefficient [59••, 68]. Ultracentrifugation is the most traditional method, but it is time-consuming [18], requires large samples [69], and results in a final product that can be contaminated with media residue or cell debris [59••]. Some studies have developed novel methodologies in order to get higher yields of EVs. Leone et al. [18] have isolated EVs from *P. fermetans* biofilm using a mammalian serum exosome kit, reporting isolations that are around 50% as effective as the ultracentrifugation method [18]. Most recently, a new protocol using solid media was proposed, taking into account the fungal colonization over solid surfaces, such as soil and plant or animal tissue. To date, this technique has been exploited for *C. neoformans*, *C. gattii*, *Candida albicans*, *H. capsulatum*, and *S. cerevisae* [59••].

A second major issue is the ongoing need to distinguish between genuine vesicles and cell artifacts. Several pieces of data indicate that fungal EVs are not cell artifacts, including TEM validation [18] and evidence that cellular debris should be eliminated from preparations by centrifugation and filtration [70]. “Mock” purifications on supernatants from heat-killed fungal cells or from media alone do not yield EVs, which can be identified only from live cells [20, 43••], and EVs show a characteristic double membrane that is unlike cell fragments [10•, 11, 54, 71]. Likewise, the striking similarity between proteomic analysis of EVs from different fungal species [7•, 11, 43••, 66] suggests that vesicle secretion is a well-conserved mechanism in fungi. Lastly, mutants of the conventional and unconventional secretion pathways produce EVs with different cargo, size, and quantitity, in comparasion with wild-type strains [53, 59••], indicating the biological origin of EVs.

One of the biggest challenges yet to be exploired is if fungal EVs are released inside host cells. Linked to this point, it is still unknown which receptors play a role on fungal EV uptake by the host cells. Antibodies against the fungal EV surface [72] or the identification of a fungal molecule as an “EV-specific marker” would help solve this question, but to date neither reagent is available.

Lastly, the field is significantly hampered by the lack of a gene modification that results in a mutant unable to secrete vesicles [72] or even a reliable way to discriminate between different vesicle populations. It is still unclear whether the biogenesis of EVs influences their cargo and function [35]. In this context, the enhancement of EVs isolation and analysis methodologies is critical for the development of the field and for the potential applications for fungal EVs, which will be discussed in the next session.

Applications and Future Perspectives

Clinically, host-derived EVs are already under development as biomarkers [73]. An exciting next step would be to use a similar approach to diagnose fungal diseases. Proteins from *C. neoformans* EVs were recognized by human serum from patients diagnosed with cryptococcosis, but not from healthy individuals [7•]. Similarly, protein extracts of *H. capsulatum* EVs reacted with serum from patients with histoplasmosis [11], and EVs from *P. brasiliensis* reacted strongly against anti-α-Gal IgG from patients with paracoccidioidomycosis [65]. A reliable EV-based diagnostic would dramatically reduce the time to diagnosis several fungal pathogens and would be particularly helpful for those organisms for which there is no available molecular test currently on the market [74].

EVs from fungal cells could potentially be also used as vaccine antigens or adjuvants, since several components (such as Bgl2 in *C. albicans*) stimulate an immune response and can confer protection against invasive candidiasis infection in mice [62]. In bacteria, outer membrane vesicles (OMV) have been highlighted as part of a vaccine strategy [75]. OMV meningococcal vaccine has been approved by regulatory agencies [76], and shown to also protect against gonorrhea [77]. A similar approach may be extremely worthwhile for fungal pathogens.

Lastly, vesicle mechanisms of secretion could be used as a potential antifungal target. Mutants of the unconventional secretion pathway, such as *apt1Δ*, were reported to be hypovirulent and unable to disseminate to the brain in mice models [53, 78]. Additionaly, EVs could be used as vehicle to deliver drug molecules to specific target tissues [79].

Summary

Despite huge recent advances in fungal EV biology, there are many important aspects that remain to be explored, such as the production of EV markers and the development of more accurate techniques for EV isolation and characterization. However, given that the fungal EV field is less than 15 years old, this is undoubtedly an area with many more exciting discoveries to come.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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- •• Of major importance

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