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Inside-out: from endosomes to extracellular vesicles in fungal RNA transport

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Abstract

Membrane-coupled RNA transport is an emerging theme in fungal biology. This review focuses on the RNA cargo and mechanistic details of transport via two inter-related sets of organelles: endosomes and extracellular vesicles for intra- and intercellular RNA transfer. Simultaneous transport and translation of messenger RNAs (mRNAs) on the surface of shuttling endosomes is a conserved process pertinent to highly polarised eukaryotic cells, such as hyphae or neurons. Here we detail the endosomal mRNA transport machinery components and mRNA targets of the core RNA-binding protein Rrm4. Extracellular vesicles (EVs) are newly garnering interest as mediators of intercellular communication, especially between pathogenic fungi and their hosts. Landmark studies in plant-fungus interactions indicate EVs as a means of delivering various cargos, most notably small RNAs (sRNAs), for cross-kingdom RNA interference. Recent advances and implications of the nascent field of fungal EVs are discussed and potential links between endosomal and EV-mediated RNA transport are proposed.

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1. Introduction

RNA molecules figure fundamentally in mediating protein production from the genetic blueprint. They serve both as components of the translation machinery as well as adaptable regulators. In this review, we focus on messenger RNAs (mRNAs) and small regulatory RNAs (sRNAs), transported in association with intracellular and extracellular organelles, namely endosomes and extracellular vesicles (EVs).

A molecule of mRNA contains, apart from the protein-coding sequence, cis-acting regulatory elements for interaction with cognate trans-acting factors. These fine-tune timing, localisation, and amplitude of translation in a combinatorial manner. Thus, each mRNA molecule interacts with various factors during its lifetime (Eliscovich and Singer, 2017; Singh et al., 2015), including small RNAs (sRNAs) and a plethora of RNA-binding proteins (Hentze et al., 2018).

Small RNAs regulate gene expression at the transcriptional and post-transcriptional level in a process known as RNA silencing or RNA interference (RNAi) in eukaryotes (Bologna and Voinnet, 2014; Chang et al., 2012; Wilson and Doudna, 2013). Dicer-like proteins (DCR, Drosha, DCL) are core factors in sRNA biogenesis that process double-strand RNA precursors into mature 20-30 nucleotide (nt) duplex sRNAs, which include microRNAs (miRNAs), small-interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs). The guide strand of sRNAs is loaded into an active Argonaute (AGO) core of the RNA-induced silencing complex (RISC) to direct sequence-specific gene silencing.

RNA-binding proteins (RBPs) contain designated domains to interact with specific elements in target RNAs. For example, the RNA recognition motifs (RRMs) of the poly(A)-binding protein recognises the poly(A) tail of almost all mRNAs (Brambilla et al., 2019; Hogan et al., 2008). Conversely, RNA elements with defined secondary and tertiary structures are bound by specific RNA-binding proteins that influence the stability, functionality and localisation of RNA molecules. Pertinent to intracellular RNA transport are complexes containing RBPs that link

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them to molecular motors to determine where and when the mRNA should be translated (Martin and Ephrussi, 2009; Niessing et al., 2018).

In recent years, a close link between RNA transport and membrane trafficking has become apparent (Béthune et al., 2019; Jansen et al., 2014). Endosomes, for example, carry mRNA along the microtubule cytoskeleton (Baumann et al., 2012). Moreover, translation of mRNA on the surface of mobile endosomes has been demonstrated as a novel mechanism to load protein cargo on endosomes for long distance transport (Baumann et al., 2012; Haag et al., 2015).

Another emerging theme is extracellular vesicle (EV)-mediated RNA transport. Various RNA species have been found in the lumen of EVs that may participate in intercellular communication. In light of the breakthrough discoveries of cross-kingdom RNAi between pathogenic fungi and their host plants (Nowara et al., 2010; Weiberg et al., 2013), EVs are emerging as probable vehicles mediating this process (Cai et al., 2018). The membrane-associated RBPs, such as the endosome-associated RNAi components (Gibbings et al., 2009; Lee et al., 2009) are predicted to facilitate selective targeting of RNA cargo into extracellular vesicles. Here, we summarise the current knowledge and carefully speculate on the mechanism of endosomal and EV-mediated RNA transport in fungi, with respect to their development and lifestyle: from endosomal transport of mRNA during polar growth of hyphae to secretion of sRNA in extracellular vesicles at the fungal-plant interface.

2. Endosomal mRNA transport

2.1 Fungal endosomes on the move

The endosomal pathway is an evolutionarily conserved membrane trafficking mechanism important for recycling and degradation of plasma membrane proteins. Starting with endocytosis, early endosomes are formed by inward budding of the plasma membrane and mature into late endosomes. Along the path of maturation, intraluminal vesicles bud inwards

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forming multivesicular endosomes (MVEs) (Huotari and Helenius, 2011). Maturing endosomes have different fates: they fuse with the vacuole for cargo degradation or they fuse with the plasma membrane, releasing its luminal contents. The intraluminal vesicles of MVEs are released as exosomes. Important regulators of intracellular membrane trafficking are small GTPases, specific subsets of which mark membrane compartment identity. Early and late endosomes, for example, are associated with Rab5- and Rab7-type GTPases, respectively (Huotari and Helenius, 2011).

Among the best-studied examples for endosomal transport in fungi is the basidiomycete *Ustilago maydis* (Haag et al., 2015; Steinberg, 2012). This corn pathogen switches from yeast-like budding to unipolar growth in order to form infectious hyphae for plant colonisation (Lanver et al., 2017). Prior to invading the plant, the cell cycle is temporarily arrested and hyphae begin to grow with a defined axis of polarity. The hyphae expand at the apical pole and insert septa at the basal pole resulting in the formation of regularly spaced empty sections that collapse over time (Fig. 1A; Vollmeister et al., 2012;). Studying endocytosis during this phase of the life cycle uncovered extensive bidirectional movement of Rab5a-positive early endosomes along microtubules (Fig. 1C-D; Steinberg, 2012, 2014). Endosomal shuttling is achieved by the concerted action of the plus end-directed Kinesin-3-type motor Kin3 towards the hyphal tip and the minus end-directed motor dynein Dyn1/2 towards the central nucleus. Loss of Kin3 results in the formation of aberrant bipolar hyphae, suggesting that endosomal transport is needed for efficient unipolar hyphal growth (Schuster et al., 2011). It has been speculated that endosomes deliver cargo proteins to the basal vacuole or transport signalling components over long distances to allow communication between the nucleus and the growing apex (Bielska et al., 2014; Steinberg, 2012, 2014).

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2.2 Rrm4: a major RBP for mRNA transport on endosomes

An insightful addition to the picture of the endosomal distribution chain was the presence of the mRNA-binding protein Rrm4 on Rab5-positive endosomes, uncovering a novel mechanism of mRNA transport in polarised cells (Baumann et al., 2012; Jansen et al., 2014). Prior to this discovery, there was genetic evidence linking Rrm4 to endosomal function and cell polarity: loss of Rrm4 leads to the formation of aberrant bipolar hyphae, similar to those of *kin3Δ* strains (Fig. 1B; Becht et al., 2006).

Rrm4 contains three RRM domains for RNA binding at the N-terminus and two MLLE domains for protein-protein interaction at the C-terminus. A recent transcriptome-wide search for sequences bound by Rrm4, down to single-nucleotide resolution, showed groups of transcripts with different patterns of Rrm4 binding along the mRNA, at the start or stop codons, the ORF, and most prominently, the 3´ untranslated region (UTR) (Fig. 1D; Olgeiser et al., 2019). We speculate that differential binding specificities of the three RRM domains, in combination with other protein interactors, bring about different binding patterns on the target mRNA. Supporting this notion, the third RRM domain recognizes the sequence motif UAUG. Furthermore, the small glycin-rich RNA-binding protein Grp1 was found to share targets Rrm4 particularly in 3´ UTRs (Fig. 1D; Olgeiser et al., 2019). In essence, the key RNA-binding protein of endosomal mRNA transport binds distinct translational landmark sites to orchestrate transport and translation.

2.3 On-the-go translation of mRNAs on shuttling endosomes

Evidence from RNA live imaging with *in vivo* UV crosslinking revealed that Rrm4 binds a distinct set of target mRNAs, including those encoding septins (König et al., 2009). Septins are cytoskeletal proteins that assemble into heteromeric building blocks, important for cell polarity and morphology (Mostowy and Cossart, 2012). In hyphae, septins form higher-order structures,

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such as filaments, with a gradient emanating from the hyphal growth pole (Fig. 1D; Baumann et al., 2014; Zander et al., 2016).

Intriguingly, the septin proteins too, were found to be present on Rrm4-positive transport endosomes, along with septin mRNA. Moreover, Rrm4-dependent shuttling of tagged ribosomes on these endosomes strongly suggests on-the-go translation of the cargo mRNA on endosomal surface (Baumann et al., 2014; Higuchi et al., 2014). Consistently, all four septin mRNAs carry Rrm4 binding sites in their 3´ untranslated region (UTR), presumably so that the binding of Rrm4 does not interfere with translation during transport (Olgeiser et al., 2019). In the absence of Rrm4, shuttling of both septin mRNA and proteins was lost, as well as septin heteromer assembly and the formation of a gradient of higher order septin filaments (Fig. 1C-D; Baumann et al., 2014; Zander et al., 2016). Thus, the novel concept of endosomal transport-coupled translation was introduced (Baumann et al., 2014): local translation and assembly of protein complexes at the surface of motile endosomes allows the efficient delivery of ready-made products to the hyphal growth pole (Fig. 1D-E).

2.4 The endosomal RNA transport machinery

A major research question surrounding Rrm4-mediated endosomal mRNA transport is how the Rrm4-containing mRNPs are attached to endosomes. Initially, it was found that mutations in critical residues of the C-terminal MLLE domain caused loss of Rrm4 movement (Becht et al., 2006). The 70-amino-acid MLLE domain was first found in the human poly(A)-binding protein PABC1. It specifically interacts with the PAM2 peptide motif (PABP interacting motif 2), present in cognate protein interaction partners (Kozlov et al., 2010; Xie et al., 2014). Search for PAM2 motif proteins lead to Upa1 (*Ustilago* PAM2 protein 1; Pohlmann et al., 2015), which additionally contains a FYVE zinc finger for the interaction with PI3P lipids characteristic for early endosomes (Kutateladze, 2006; Stenmark et al., 2002). Indeed, Upa1 shuttles on almost all Rrm4-positive endosomes and the loss of Upa1 causes aberrant bipolar hyphal growth. Upa1

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interacts with Rrm4 but unexpectedly, the PAM2 motif was dispensable for this function (Pohlmann et al., 2015). Instead, it was found to contain two PAM2-like sequences (PAM2L) for interaction with the MLLE domains of Rrm4 (Pohlmann et al., 2015). Taken together, Upa1 is the first example of a functionally important adaptor protein linking Rrm4-containing mRNPs to endosomes (Fig. 1E; Pohlmann et al., 2015). However, even in the absence of Upa1, residual endosomal shuttling of Rrm4 is observed, suggesting that there are additional factors involved.

Upa2, which exceptionally contains four PAM2 motifs, shuttles on almost all Rrm4-positive endosomes and is important for efficient unipolar hyphal growth (Jankowski et al., 2019). However, in contrast to Upa1 it requires Rrm4 to be present on endosomes, indicating that it most likely interacts with the components of the mRNP, rather than directly with the endosomal membrane. Also in this case, the PAM2 motifs were functionally dispensable. Instead, a novel functionally important effector domain was discovered at the N-terminus and a conserved GWW motif for endosomal mRNP attachment at the C-terminus. Loss of Upa2 did not influence Rrm4, but shuttling of the poly(A)-binding protein Pab1 and specific target mRNAs was strongly reduced. Thus, Upa2 classifies as a novel core component of endosomal mRNA transport, which most likely serves as a scaffold protein for endosomal mRNP assembly or stability during transport (Fig. 1E; Jankowski et al., 2019).

To learn more about the identity of the transport endosomes, we studied the conserved factor Did2, which regulates the ESCRT machinery (endosomal sorting complex required for transport) for endosomal maturation (Hurley, 2015; Teis et al., 2009). Loss of Did2 caused aberrant bipolar hyphal growth, suggesting a link to endosomal mRNA transport. Closer inspection revealed that maturation of shuttling endosomes was indeed disturbed, since marker proteins Rab7 or vacuolar cargo proteins were present on shuttling endosomes in *did2Δ* hyphae (Haag et al., 2015). The altered identity of the shuttling endosomes causes reduced attachment of the motor Kin3 as well as less FYVE protein Upa1. Consequently, mRNPs were transported

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less efficiently, explaining the phenotype. Thus, the ESCRT regulator orchestrates the balance of early endosomes functioning in long-distance transport and endocytic maturation (Haag et al., 2017).

2.5 Membrane-associated RNA transport as a widespread concept

Membrane-associated RNA transport appears to be a common theme in biology (Béthune et al., 2019). Within the fungal kingdom, a detailed phylogenetic analysis of the core endosomal RNA transport machinery components revealed their conservation across Basidiomycota and absence in Ascomycota (Müller et al., 2019). Endosomal shuttling of the heterologous expressed Rrm4 orthologue from fungi as distant as *Rhizophagus irregularis* in *U. maydis*, suggests a high degree of functional conservation (Müller et al., 2019). Intriguingly, microtubule-dependent shuttling of the RNA-binding protein Gul1 was recently reported in hyphae of the ascomycete *Neurospora crassa*, although the mode of membrane association is still unclear (Herold et al., 2019).

Comparable to endosomal mRNA transport in *U. maydis*, neuronal endosomes were discovered to deliver mRNAs and promote mitochondrial targeting of nuclear encoded proteins by local translation at the surface of late endosomes (Cioni et al., 2019). Furthermore, the mammalian RNA-binding protein ANXA11 links RNA granules to moving lysosomes for long-distance mRNA transport in neurons (Liao et al., 2019). In essence, endosomal mRNA transport is not an exceptional invention in basidiomycete smut fungi, but a widespread trafficking process.

On a wider scale, membrane-associated RNA-binding proteins (memRBPs) coordinate membrane-coupled local translation, not only at endosomes or the ER but most likely at all internal membranes including those of mitochondria, peroxisomes and vacuoles (Béthune et al., 2019). This brings us to hypothesise that such memRBPs would also facilitate specific loading of various RNA cargo from intercommunicating intracellular organelles into secreted

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extracellular membrane structures, which can be considered “extended” organelles that can bring about extended phenotypes (Dawkins, 1982).

3 Extracellular vesicle-mediated RNA transport

3.1 Extracellular vesicles

Extracellular vesicles (EVs) are membranous nano-sized particles secreted by organisms representing the kingdoms of life. Despite initial disregard as being cell debris or disposals, cumulative evidence clearly indicates biological functionality of EVs, particularly in intercellular and inter-organismal communication (Deatherage and Cookson, 2012; Maas et al., 2017; Meldolesi, 2018; Mittelbrunn and Sanchez-Madrid, 2012). EVs are now recognized as common vehicles that deliver molecules such as RNAs and proteins to instigate physiological changes in recipient cells. Already observed in early ultrastructural studies, EVs have only recently begun to gain increasing attention from plant scientists and microbiologists. EVs are proposed to play pivotal roles in cross-kingdom communication between microbial pathogens and their hosts (Bielska et al., 2019; Bielska and May, 2019; Kuipers et al., 2018; Rutter and Innes, 2018; Rybak and Robatzek, 2019; Samuel et al., 2015; Soares et al., 2017). In this part, we summarise the state-of-the-art in fungal EVs, their protein and RNA cargos as well as their potential function in intra-species to cross-kingdom communication.

3.2 EV biogenesis in fungi

EVs are a collective term for a very heterogeneous group of lipid bilayer particles varying in size, composition and cargo. Such high level of heterogeneity suggests that distinct EV biogenesis pathways must exist in cells (Mathieu et al., 2019; van Niel et al., 2018). In mammalian cell types, two major EV secretion mechanisms have been described. On the one hand, intraluminal vesicles in multivesicular endosomes (MVEs) are released as exosomes upon fusion of MVEs with the plasma membrane (Fig. 2). On the other hand, microvesicles

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bud directly off the plasma membrane, which explains the overlap in molecular contents in this type of EVs with the local cytoplasm at the cell periphery. In both EV secretion pathways, conserved ESCRT components and accessory proteins are involved (Colombo et al., 2013). Furthermore, various proteins that are linked to endomembrane systems, such as small GTPases (Muralidharan-Chari et al., 2009), SNAREs (Fader et al., 2009; Koles et al., 2012), syntenins (Baietti et al., 2012) and tetraspanins (van Niel et al., 2011), are relevant for EV biogenesis and cargo loading. Homologous proteins and similar secretory pathways are likely to participate in fungal EV biogenesis as well, but their relative contribution and biological significance remain to be clarified (Oliveira et al., 2013). In this regard, genetic evidence suggests involvement of both the conventional secretory pathway and the ESCRT-mediated MVE pathway in fungal EV biogenesis and cargo loading. For instance, *Saccharomyces cerevisiae* mutants of both the exocytic Rab GTPase *Sec4*, required for post-Golgi secretory vesicle formation, and the ESCRT component *Snf7,* show altered EV protein composition (Oliveira et al., 2010b). Furthermore, knocking down the exocyst component *Sec6* in the fungus *Cryptococcus neoformans* led to a dramatic reduction in EV secretion (Panepinto et al., 2009), presumably by affecting MVE fusion with the plasma membrane. Obviously, disruption of individual genes involved in EV biogenesis does not completely abolish EV formation, implying a certain level of functional redundancy of genes and pathways in EV formation.

3.3 Proteins and RNAs in fungal EVs

To gain further insights into the biogenesis of fungal EVs and their potential roles in fungal biology and pathogenicity, several studies have examined the EV protein and RNA cargos (Rodrigues et al., 2014). Commonly, many proteins found in EVs indeed lack classical signal peptides, supporting their cellular release via unconventional secretion mechanisms (Rodrigues et al., 2008). Moreover, comparative proteomics of fungal EVs displayed not only high diversity, but also revealed core sets of cargo proteins, indicating some degree of conservation

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in EV biogenesis, cargo loading and function (Rodrigues et al., 2014; Vallejo et al., 2012). These EV core proteins were predicted to function in translation, carbohydrate and protein metabolism, oxidation/reduction, transport, stress response and signalling functions (Vallejo et al., 2012). Moreover, several virulence factors have been found in EVs of pathogenic fungal species, suggesting a role of EVs in pathogenesis (Bleackley et al., 2019).

Beside proteins, several RNA species have been identified in fungal EVs. To date, studies on various fungal species have predominantly focused on smaller non-coding RNAs (<200 nt), including potential gene-regulatory small RNAs, such as miRNA-like RNAs (milRNAs) and tRNA fragments (tRFs) (Fig. 2; Alves et al., 2019; Peres da Silva et al., 2019; Peres da Silva et al., 2015; Rayner et al., 2017). The detection of small RNAs in fungal EVs supports their proposed role in RNA-mediated intra- or interspecific communication. Moreover, full-length mRNAs have also been found in fungal EVs (Alves et al., 2019; Peres da Silva et al., 2019), but it needs to be clarified whether EV mRNAs are translated into functional peptides in recipient cells. Beside detection, enrichment of certain RNA species and sequence motifs has been reported in plant and animal EVs (Villarroya-Beltri et al., 2014). Indeed, there seems to be clear differences between cellular and EV abundance of transcripts (Alves et al., 2019; Peres da Silva et al., 2019) implying the existence of active, yet unknown RNA sorting mechanisms into EVs. In this regard, RNA-binding proteins that form ribonucleoprotein complexes were found to facilitate loading of specific microRNAs into mammalian exosomes (Statello et al., 2018; Villarroya-Beltri et al., 2014). Similarly, ribonucleoprotein complexes are prime suspects to mediate RNA sorting into fungal EVs, as well (Fig.2). Accordingly, candidate RBPs have been detected in fungal EV proteome studies (Alves et al., 2019), thus waiting to be studied for their role in EV RNA sorting.

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3.4 EVs in human-pathogenic fungi

Fungal EVs are thought to participate in intercellular communication regarding host-fungal or fungal-microbial interactions. Indeed, fungal EVs released from different pathogenic species can either support host infection (Bielska et al., 2018; Ikeda et al., 2018) or stimulate immune responses in their mammalian host cells (Oliveira et al., 2010a; Vargas et al., 2015). For instance, EVs isolated from the culture supernatant of *Candida albicans* or *Cryptococcus neoformans* have immunomodulatory effects on macrophages and other immune cells (Joffe et al., 2016; Zamith-Miranda et al., 2018). Known virulence-associated proteins, such as laccases and ureases, were found in *C. neoformans* and *C. albicans* EVs, suggesting vesicular transport of such virulence factors towards host cells for infection (Oliveira et al., 2010b; Rodrigues et al., 2008). Other non-proteinaceous compounds were also detected in fungal EVs that are known to contribute to pathogenicity and virulence, such as melanin and the polysaccharide glucuronoxylomannan (Eisenman et al., 2009; Rodrigues et al., 2007). Interestingly, fungi do not only secrete EVs for pathogenesis, but eventually also for defence against predators. For instance, *C. neoformans* was reported to release EVs for protection against the predatory amoeba *Acanthamoeba castellanii*. The fungal EVs are internalised by the amoeba cells and are suggested to suppress predatory activity that result in increased fungal survival rates (Rizzo et al., 2017).

An interesting function of fungal EVs has been proposed in regard to intraspecific, intercellular communication at the population level (Bielska and May, 2019). Virulence of the *Cryptococcus gattii* outbreak lineage R265 is attributed to an explosive proliferative ability through “division of labour” between fungal cells co-infecting a macrophage (Voelz et al., 2014). In this context, EVs isolated from axenic culture of the outbreak strain are sufficient to trigger rapid proliferation of a recipient non-outbreak strain inside macrophages in cell culture. Interestingly, both the EV protein and RNA cargoes are essential for this effect. Proliferation of the non-

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outbreak strain in macrophages in the presence of other macrophages infected with the outbreak strain further supports EV-mediated long-distance communication (Bielska et al., 2018). Similarly, bacterial outer membrane vesicles were also reported to transport quorum sensing molecules (Toyofuku, 2019), indicating that EVs may be a common means of microbial communication at population level.

3.5 EVs in plant-fungal interactions

EV- and MVE-like structures have been also observed in plants by microscopic techniques at infection sites of fungal pathogens (Fig. 2; An et al., 2007; Snetselaar and Mims, 1994). Ultrastructural examination of non-host interaction between the barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei* and *Arabidopsis thaliana* revealed plant MVEs and syntaxin PEN1-positive exosomes accumulating around the fungal infection structures (An et al., 2006; Böhlenius et al., 2010; Meyer et al., 2009). Intriguingly, an antimicrobial capacity of infection-induced PEN1-positive EVs was proposed recently; EVs isolated from leaf apoplastic wash fluids of *Arabidopsis* plants challenged with the bacterial pathogen *Pseudomonas syringae* showed enrichment of antimicrobial peptides, such as Pathogenesis-Related (PR) proteins (Hansen and Nielsen, 2017; Rutter and Innes, 2017). Plant EVs were found to supress fungal pathogens also. For instance, incubation of *Sclerotinia sclerotiorum* liquid culture with EVs isolated from sunflower apoplastic wash fluid led to uptake of plant EVs by the fungus and subsequent growth inhibition (Regente et al., 2017). However, the identity of the components of plant EVs inhibiting fungal proliferation remains unknown. *Arabidopsis* EVs also contain different types of small and tiny RNAs (Baldrich et al., 2019) that might mediate plant-pathogen crosstalk. The phenomenon, whereby plant host-derived sRNA silences genes in the pathogen, is known as host-induced gene silencing (HIGS; Fig. 2; Nowara et al., 2010). Recently, it was demonstrated for the first time in *Arabidopsis*, that HIGS is mediated by EVs for plant defence (Huang et al., 2019). *Arabidopsis* delivers miRNAs and trans-acting siRNAs (tasiRNAs) via

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exosome-like EVs into cells of the fungal plant pathogen *Botrytis cinerea* during infection. EV sRNAs were found to suppress fungal virulence genes putatively involved in intracellular transport and pathogenesis (Cai et al., 2018). Along the same lines, *Arabidopsis* EVs are proposed to also deliver siRNAs into the oomycete plant pathogen *Phytophthora capsici*, possibly to silence virulence genes (Hou et al., 2019). Likewise, cotton plants also deliver miRNAs to the fungal pathogen *Verticillium dahliae* to inhibit virulence gene expression and to promote disease resistance (Zhang et al., 2016), but participation of cotton EVs in miRNA transport has so far not been examined.

Cross-kingdom RNAi in plant-fungal interaction is bidirectional (Wang et al., 2016), because pathogen-induced gene silencing (PIGS; Fig. 2) by a fungal pathogen has been initially discovered as a virulence strategy of *B. cinerea*. This fungal pathogen delivers sRNAs into plant cells during infection, which hijack the plant RNAi machinery to silence host immunity genes (Weiberg et al., 2013). Similarly, sRNAs of the fungal plant pathogen *Verticillium dahliae* were found associated with the plant RNAi machinery during infection (Wang et al., 2016). Moreover, miRNA-like RNAs of the wheat pathogens *Puccinia striiformis f.sp. tritici* and *Fusarium graminearum* were suggested to target host plant genes for infection (Wang et al., 2017, Jian and Liang, 2019). Other types of plant pathogens, parasites or symbionts are proposed to deliver sRNAs into their host plants to manipulate gene expression (Weiberg et al., 2015). Indeed interspecies and cross-kingdom RNAi has been discovered in the parasitic plant *Cuscuta spp*. (Johnson and Axtell, 2019) and the nitrogen-fixing bacteria *Sinorhizobium meliloti* (Ren et al., 2019). Whether fungi and other microbes deliver sRNAs and other types of virulence factors (effectors) into host cells via EVs, needs to be resolved. Yet, another type of membranous structure, called membrane tubules (“memtubs”), has been described at the interface between the arbuscular mycorrhizal fungus *Rhizophagus irregularis* and its plant host (Ivanov et al., 2019; Roth et al., 2019). Memtubs seem to be generally conserved in plant-

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fungus interaction, and have also been observed in the pathogen *U. maydis* (Roth et al., 2019). Consistent with the hypothesis, memtubs might be produced to increase surface areas for exchange of signals and nutrients at the fungus-plant interface. However, any functional role of memtubs and whether RNAs and proteins can be transported via this route between fungi and plants needs to be investigated.

How EVs of 50-500 nm in diameter can traverse the cell wall of bacteria, fungi or plants is currently poorly understood. Different models of vesicular trans-cell wall shuttling have been postulated (Brown et al., 2015; Wolf and Casadevall, 2014). One hypothesis is that EVs cross the cell wall via pores or channels (Brown et al., 2015 Walker et al., 2018). However, electron microscopy studies of EV interaction with the fungal cell wall in *C. neoformans* suggest direct vesicular exit through mechanisms that depend on cell wall melanisation (Wolf et al., 2014), indicating that cell wall composition matters. Similarly, the viscoelastic properties of *C. albicans* cell walls seem to influence the traffic of liposomes (Walker et al., 2018). Higher cell wall plasticity at the site of cell separation, hyphal branching or actively growing daughter cells and hyphal tips may facilitate EV release as well. Interestingly, many putative cell wall remodelling enzymes, such as glucanases and pectinases were identified in EVs, suggesting cell wall modifying activity by EVs may promote their cell wall passage (Nimrichter et al., 2016; Rodrigues et al., 2014).

4. Concluding remarks

As outlined above, membrane and RNA trafficking are two tightly intertwined processes. A key unanswered question is how transported RNAs are specifically loaded to membrane compartments. This includes the attachment of mRNAs to the surface of endosomes and the loading of extracellular vesicles with RNA cargo. Determining factors are most likely RNA-binding proteins that interact with membrane-associated proteins. The precise significance of membrane-coupled RNA transport in localised subcellular processes as well as intercellular

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communication shaping fungal populations, fungus-host interactions, and even the greater microbiome, remains to be elucidated. As so often, fungi could serve as excellent model systems to advance this emerging research area.

Conflict of interest

All authors agree with the submission and declare no conflict of interest.

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References

Alves, L. R., Peres da Silva, R., Sanchez, D. A., Zamith-Miranda, D., Rodrigues, M. L., Goldenberg, S., Puccia, R., Nosanchuk, J. D., 2019. Extracellular vesicle-mediated RNA release in *Histoplasma capsulatum*. mSphere 4, e00176-00119.

An, Q., Huckelhoven, R., Kogel, K. H., van Bel, A. J., 2006. Multivesicular bodies participate in a cell wall-associated defence response in barley leaves attacked by the pathogenic powdery mildew fungus. Cell. Microbiol. 8, 1009-1019.

 doi:10.20944/preprints201911.0213.v1

An, Q., van Bel, A. J., Huckelhoven, R., 2007. Do plant cells secrete exosomes derived from multivesicular bodies? Plant Signal Behav. 2, 4-7.

Baietti, Maria Francesca, Zhang, Zhe, Mortier, Eva, Melchior, Aurélie, Degeest, Gisèle, Geeraerts, Annelies, Ivarsson, Ylva, Depoortere, Fabienne, Coomans, Christien, Vermeiren, Elke, Zimmermann, Pascale, David, Guido, 2012. Syndecan–syntenin–ALIX regulates the biogenesis of exosomes. Nat. Cell Biol. 14, 677.

Baldrich, P., Rutter, B. D., Karimi, H. Z., Podicheti, R., Meyers, B. C., Innes, R. W., 2019. Plant extracellular vesicles contain diverse small RNA species and are enriched in 10- to 17-nucleotide "tiny" RNAs. Plant Cell 31, 315-324.

Baumann, S., König, J., Koepke, J., Feldbrügge, M., 2014. Endosomal transport of septin mRNA and protein indicates local translation on endosomes and is required for correct septin filamentation. EMBO Rep. 15, 94-102.

Baumann, S., Pohlmann, T., Jungbluth, M., Brachmann, A., Feldbrügge, M., 2012. Kinesin-3 and dynein mediate microtubule-dependent co-transport of mRNPs and endosomes. J. Cell Sci. 125, 2740-2752.

Becht, P., König, J., Feldbrügge, M, 2006. The RNA-binding protein Rrm4 is essential for polarity in *Ustilago maydis* and shuttles along microtubules. J. Cell Sci. 119, 4964-4973.

Béthune, J., Jansen, R. P., Feldbrügge, M., Zarnack, K., 2019. Membrane-associated RNA-binding proteins orchestrate organelle-coupled translation Trends Cell Biol. 29, 178-188.

Bielska, E., Birch, P. R. J., Buck, A. H., Abreu-Goodger, C., Innes, R. W., Jin, H., Pfaffl, M. W., Robatzek, S., Regev-Rudzki, N., Tisserant, C., Wang, S., Weiberg, A., 2019. Highlights of the mini-symposium on extracellular vesicles in inter-organismal communication, held in Munich, Germany, August 2018. J. Extracell. Vesicles 8, 1590116.

Bielska, E., Higuchi, Y., Schuster, M., Steinberg, N., Kilaru, S., Talbot, N. J., Steinberg, G., 2014. Long-distance endosome trafficking drives fungal effector production during plant infection. Nat. Commun. 5, 5097.

 doi:10.20944/preprints201911.0213.v1

Bielska, E., May, R. C., 2019. Extracellular vesicles of human pathogenic fungi. Curr. Opin. Microbiol. 52, 90-99.

Bielska, E., Sisquella, M. A., Aldeieg, M., Birch, C., O'Donoghue, E. J., May, R. C., 2018. Pathogen-derived extracellular vesicles mediate virulence in the fatal human pathogen *Cryptococcus gattii*. Nat. Commun. 9, 1556.

Bleackley, M. R., Dawson, C. S., Anderson, M. A., 2019. Fungal extracellular vesicles with a focus on proteomic analysis. Proteomics. 19, e1800232.

Böhlenius, H., Morch, S. M., Godfrey, D., Nielsen, M. E., Thordal-Christensen, H., 2010. The multivesicular body-localized GTPase ARFA1b/1c is important for callose deposition and ROR2 syntaxin-dependent preinvasive basal defense in barley. Plant Cell 22, 3831-3844.

Bologna, N. G., Voinnet, O., 2014. The diversity, biogenesis, and activities of endogenous silencing small RNAs in Arabidopsis. Annu. Rev. Plant Biol. 65, 473-503.

Brachmann, A., Weinzierl, G., Kämper, J., Kahmann, R., 2001. Identification of genes in the bW/bE regulatory cascade in *Ustilago maydis*. Mol. Microbiol. 42, 1047-1063.

Brambilla, M., Martani, F., Bertacchi, S., Vitangeli, I., Branduardi, P., 2019. The *Saccharomyces cerevisiae* poly (A) binding protein (Pab1): Master regulator of mRNA metabolism and cell physiology. Yeast 36, 23-34.

Brown, L., Wolf, J. M., Prados-Rosales, R., Casadevall, A., 2015. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nat. Rev. Microbiol. 13, 620-630.

Cai, Q., Qiao, L., Wang, M., He, B., Lin, F. M., Palmquist, J., Huang, S. D., Jin, H., 2018. Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. Science 360, 1126-1129.

Chang, S. S., Zhang, Z., Liu, Y., 2012. RNA interference pathways in fungi: mechanisms and functions. Annu. Rev. Microbiol. 66, 305-323.

 doi:10.20944/preprints201911.0213.v1

Cioni, J. M., Lin, J. Q., Holtermann, A. V., Koppers, M., Jakobs, M. A. H., Azizi, A., Turner-Bridger, B., Shigeoka, T., Franze, K., Harris, W. A., Holt, C. E., 2019. Late endosomes act as mRNA translation platforms and sustain mitochondria in axons. Cell 176, 56-72 e15.

Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., Manel, N., Moita, L. F., Thery, C., Raposo, G., 2013. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. J. Cell Sci. 126, 5553-5565.

Dawkins, R. 1982. The extended phenotype. Oxford, UK: Oxford University Press.

Deatherage, B. L., Cookson, B. T., 2012. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. Infect. Immun. 80, 1948-1957.

Eisenman, H. C., Frases, S., Nicola, A. M., Rodrigues, M. L., Casadevall, A., 2009. Vesicle-associated melanization in *Cryptococcus neoformans*. Microbiology 155, 3860-3867.

Eliscovich, C., Singer, R. H., 2017. RNP transport in cell biology: the long and winding road. Curr. Opin. Cell. Biol. 45, 38-46.

Fader, C. M., Sanchez, D. G., Mestre, M. B., Colombo, M. I., 2009. TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. Biochim. Biophys. Acta 1793, 1901-1916.

Gibbings, D. J., Ciaudo, C., Erhardt, M., Voinnet, O., 2009. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. Nat. Cell Biol. 11, 1143-1149.

Haag, C., Pohlmann, T., Feldbrugge, M., 2017. The ESCRT regulator Did2 maintains the balance between long-distance endosomal transport and endocytic trafficking. PloS Genet. 13, e1006734.

 doi:10.20944/preprints201911.0213.v1

Haag, C., Steuten, B., Feldbrügge, M., 2015. Membrane-coupled mRNA trafficking in fungi. Annu. Rev. Microbiol. 69, 265-281.

Hansen, L. L., Nielsen, M. E., 2017. Plant exosomes: using an unconventional exit to prevent pathogen entry? J. Exp. Bot. 69, 59-68.

Hentze, M. W., Castello, A., Schwarzl, T., Preiss, T., 2018. A brave new world of RNA-binding proteins. Nat. Rev. Mol. Cell. Biol. 19, 327-341.

Herold, I., Kowbel, D., Delgado-Alvarez, D. L., Garduno-Rosales, M., Mourino-Perez, R. R., Yarden, O., 2019. Transcriptional profiling and localization of GUL-1, a COT-1 pathway component, in *Neurospora crassa*. Fungal Genet. Biol. 126, 1-11.

Higuchi, Y., Ashwin, P., Roger, Y., Steinberg, G., 2014. Early endosome motility spatially organizes polysome distribution. J. Cell Biol. 204, 343-357.

Hogan, D. J., Riordan, D. P., Gerber, A. P., Herschlag, D., Brown, P. O., 2008. Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system. PLoS Biol. 6, e255.

Hou, Y., Zhai, Y., Feng, L., Karimi, H. Z., Rutter, B. D., Zeng, L., Choi, D. S., Zhang, B., Gu, W., Chen, X., Ye, W., Innes, R. W., Zhai, J., Ma, W., 2019. A *Phytophthora* effector suppresses trans-kingdom RNAi to promote disease susceptibility. Cell Host Microbe 25, 153-165 e155.

Huang, C. Y., Wang, H., Hu, P., Hamby, R., Jin, H., 2019. Small RNAs - Big players in plant-microbe interactions. Cell Host Microbe 26, 173-182.

Huotari, J., Helenius, A., 2011. Endosome maturation. EMBO J. 30, 3481-3500.

Hurley, J. H., 2015. ESCRTs are everywhere. EMBO J. 34, 2398-2407.

Ikeda, M. A. K., de Almeida, J. R. F., Jannuzzi, G. P., Cronemberger-Andrade, A., Torrecilhas, A. C. T., Moretti, N. S., da Cunha, J. P. C., de Almeida, S. R., Ferreira, K. S., 2018. Extracellular vesicles from *Sporothrix brasiliensis* are an important virulence factor that

 doi:10.20944/preprints201911.0213.v1

induce an increase in fungal burden in experimental sporotrichosis. Front. Microbiol. 9, 2286.

Ivanov, S., Austin, J., 2nd, Berg, R. H., Harrison, M. J., 2019. Extensive membrane systems at the host-arbuscular mycorrhizal fungus interface. Nat. Plants 5, 194-203.

Jankowski, S., Pohlmann, T., Baumann, S., Müntjes, K.M., Devan, S.K., Zander, S., Feldbrügge, M., 2019. The multi PAM2 protein Upa2 functions as novel core component of endosomal mRNA transport. EMBO Rep. 24, e47381.

Jansen, R. P., Niessing, D., Baumann, S., Feldbrügge, M., 2014. mRNA transport meets membrane traffic. Trends Genet. 30, 408-417.

Jian, J., Liang, X., 2019. One small RNA of *Fusarium graminearum* targets and silences CEBiP gene in common wheat. Microorg. 7, 425.

Joffe, L. S., Nimrichter, L., Rodrigues, M. L., Del Poeta, M., 2016. Potential roles of fungal extracellular vesicles during infection. mSphere 1, e00099-00016.

Johnson, N. R., Axtell, M. J., 2019. Small RNA warfare: exploring origins and function of trans-species microRNAs from the parasitic plant *Cuscuta*. Curr. Opin. Plant Biol. 50, 76-81.

Koles, K., Nunnari, J., Korkut, C., Barria, R., Brewer, C., Li, Y., Leszyk, J., Zhang, B., Budnik, V., 2012. Mechanism of evenness interrupted (Evi)-exosome release at synaptic boutons. J. Biol. Chem. 287, 16820-16834.

König, J., Baumann, S., Koepke, J., Pohlmann, T., Zarnack, K., Feldbrügge, M., 2009. The fungal RNA-binding protein Rrm4 mediates long-distance transport of *ubi1* and *rho3* mRNAs. EMBO J. 28, 1855-1866.

Kozlov, G., Menade, M., Rosenauer, A., Nguyen, L., Gehring, K., 2010. Molecular determinants of PAM2 recognition by the MLLE domain of poly(A)-binding protein. J. Mol. Biol. 397, 397-407.

Kuipers, M. E., Hokke, C. H., Smits, H. H., Nolte-'t Hoen, E. N. M., 2018. Pathogen-derived extracellular vesicle-associated molecules that affect the host immune system: an overview. Front. Microbiol. 9, 2182.

Kutateladze, T. G., 2006. Phosphatidylinositol 3-phosphate recognition and membrane docking by the FYVE domain. Biochim. Biophys. Acta. 1761, 868-877.

Lanver, D., Tollot, M., Schweizer, G., Lo Presti, L., Reissmann, S., Ma, L. S., Schuster, M., Tanaka, S., Liang, L., Ludwig, N., Kahmann, R., 2017. *Ustilago maydis* effectors and their impact on virulence. Nat. Rev. Microbiol. 15, 409-421.

Lee, Young Sik, Pressman, Sigal, Andress, Arlise P., Kim, Kevin, White, Jamie L., Cassidy, Justin J., Li, Xin, Lubell, Kim, Lim, Do Hwan, Cho, Ik Sang, Nakahara, Kenji, Preall, Jonathan B., Bellare, Priya, Sontheimer, Erik J., Carthew, Richard W., 2009. Silencing by small RNAs is linked to endosomal trafficking. Nat. Cell Biol. 11, 1150.

Liao, Y. C., Fernandopulle, M. S., Wang, G., Choi, H., Hao, L., Drerup, C. M., Patel, R., Qamar, S., Nixon-Abell, J., Shen, Y., Meadows, W., Vendruscolo, M., Knowles, T. P. J., Nelson, M., Czekalska, M. A., Musteikyte, G., Gachechiladze, M. A., Stephens, C. A., Pasolli, H. A., Forrest, L. R., St George-Hyslop, P., Lippincott-Schwartz, J., Ward, M. E., 2019. RNA granules hitchhike on lysosomes for long-distance transport, using annexin A11 as a molecular tether. Cell 179, 147-164 e120.

Maas, S. L. N., Breakefield, X. O., Weaver, A. M., 2017. Extracellular vesicles: Unique intercellular delivery vehicles. Trends Cell Biol. 27, 172-188.

Martin, K. C., Ephrussi, A., 2009. mRNA localization: gene expression in the spatial dimension. Cell 136, 719-730.

Mathieu, M., Martin-Jaular, L., Lavieu, G., Thery, C., 2019. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat. Cell Biol. 21, 9-17.

 doi:10.20944/preprints201911.0213.v1

Meldolesi, J., 2018. Exosomes and ectosomes in intercellular communication. Curr Biol 28, R435-R444.

Meyer, D., Pajonk, S., Micali, C., O'Connell, R., Schulze-Lefert, P., 2009. Extracellular transport and integration of plant secretory proteins into pathogen-induced cell wall compartments. Plant J. 57, 986-999.

Mittelbrunn, M., Sanchez-Madrid, F., 2012. Intercellular communication: diverse structures for exchange of genetic information. Nat. Rev. Mol. Cell Biol. 13, 328-335.

Mostowy, S., Cossart, P., 2012. Septins: the fourth component of the cytoskeleton. Nat. Rev. Mol. Cell Biol. 13, 183-194.

Müller, J., Pohlmann, T., Feldbrügge, M., 2019. Core components of endosomal mRNA transport are evolutionarily conserved in fungi. Fungal Genet. Biol. 126, 12-16.

Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., D'Souza-Schorey, C., 2009. ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. Curr. Biol. 19, 1875-1885.

Niessing, D., Jansen, R. P., Pohlmann, T., Feldbrügge, M., 2018. mRNA transport in fungal top models. Wiley Interdiscip. Rev. RNA 9, e1453.

Nimrichter, L., de Souza, M. M., Del Poeta, M., Nosanchuk, J. D., Joffe, L., Tavares Pde, M., Rodrigues, M. L., 2016. Extracellular vesicle-associated transitory cell wall components and their impact on the interaction of fungi with host cells. Front. Microbiol. 7, 1034.

Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., Hensel, G., Kumlehn, J., Schweizer, P., 2010. HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. Plant Cell 22, 3130-3141.

Olgeiser, L., Haag, C., Boerner, S., Ule, J., Busch, A., Koepke, J., König, J., Feldbrügge, M., Zarnack, K., 2019. The key protein of endosomal mRNP transport Rrm4 binds translational landmark sites of cargo mRNAs. EMBO Rep. 20, e46588.

 doi:10.20944/preprints201911.0213.v1

Oliveira, D. L., Freire-de-Lima, C. G., Nosanchuk, J. D., Casadevall, A., Rodrigues, M. L., Nimrichter, L., 2010a. Extracellular vesicles from *Cryptococcus neoformans* modulate macrophage functions. Infect. Immun. 78, 1601-1609.

Oliveira, D. L., Nakayasu, E. S., Joffe, L. S., Guimaraes, A. J., Sobreira, T. J., Nosanchuk, J. D., Cordero, R. J., Frases, S., Casadevall, A., Almeida, I. C., Nimrichter, L., Rodrigues, M. L., 2010b. Characterization of yeast extracellular vesicles: evidence for the participation of different pathways of cellular traffic in vesicle biogenesis. PLoS One 5, e11113.

Oliveira, D. L., Rizzo, J., Joffe, L. S., Godinho, R. M., Rodrigues, M. L., 2013. Where do they come from and where do they go: candidates for regulating extracellular vesicle formation in fungi. Int. J. Mol. Sci. 14, 9581-9603.

Panepinto, J., Komperda, K., Frases, S., Park, Y. D., Djordjevic, J. T., Casadevall, A., Williamson, P. R., 2009. Sec6-dependent sorting of fungal extracellular exosomes and laccase of *Cryptococcus neoformans*. Mol. Microbiol. 71, 1165-1176.

Peres da Silva, R., Longo, L. G. V., Cunha, Jpcd, Sobreira, T. J. P., Rodrigues, M. L., Faoro, H., Goldenberg, S., Alves, L. R., Puccia, R., 2019. Comparison of the RNA Content of Extracellular Vesicles Derived from *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*. Cells 8.

Peres da Silva, R., Puccia, R., Rodrigues, M. L., Oliveira, D. L., Joffe, L. S., Cesar, G. V., Nimrichter, L., Goldenberg, S., Alves, L. R., 2015. Extracellular vesicle-mediated export of fungal RNA. Sci. Rep. 5, 7763.

Pohlmann, T., Baumann, S., Haag, C., Albrecht, M., Feldbrügge, M., 2015. A FYVE zinc finger domain protein specifically links mRNA transport to endosome trafficking. Elife 4, e06041.

 doi:10.20944/preprints201911.0213.v1

Rayner, S., Bruhn, S., Vallhov, H., Andersson, A., Billmyre, R. B., Scheynius, A., 2017. Identification of small RNAs in extracellular vesicles from the commensal yeast *Malassezia sympodialis*. Sci. Rep. 7, 39742.

Regente, M., Pinedo, M., San Clemente, H., Balliau, T., Jamet, E., de la Canal, L., 2017. Plant extracellular vesicles are incorporated by a fungal pathogen and inhibit its growth. J. Exp. Bot. 68, 5485-5495.

Ren, B., Wang, X., Duan, J., Ma, J., 2019. Rhizobial tRNA-derived small RNAs are signal molecules regulating plant nodulation. Science 365, 919-922.

Rizzo, J., Albuquerque, P. C., Wolf, J. M., Nascimento, R., Pereira, M. D., Nosanchuk, J. D., Rodrigues, M. L., 2017. Analysis of multiple components involved in the interaction between *Cryptococcus neoformans* and *Acanthamoeba castellanii*. Fungal Biol. 121, 602-614.

Rodrigues, M. L., Nakayasu, E. S., Almeida, I. C., Nimrichter, L., 2014. The impact of proteomics on the understanding of functions and biogenesis of fungal extracellular vesicles. J. Proteomics 97, 177-186.

Rodrigues, M. L., Nakayasu, E. S., Oliveira, D. L., Nimrichter, L., Nosanchuk, J. D., Almeida, I. C., Casadevall, A., 2008. Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. Eukaryot. Cell 7, 58-67.

Rodrigues, M. L., Nimrichter, L., Oliveira, D. L., Frases, S., Miranda, K., Zaragoza, O., Alvarez, M., Nakouzi, A., Feldmesser, M., Casadevall, A., 2007. Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. Eukaryot. Cell 6, 48-59.

Roth, R., Hillmer, S., Funaya, C., Chiapello, M., Schumacher, K., Lo Presti, L., Kahmann, R., Paszkowski, U., 2019. Arbuscular cell invasion coincides with extracellular vesicles and membrane tubules. Nat. Plants 5, 204-211.

 doi:10.20944/preprints201911.0213.v1

Rutter, B. D., Innes, R. W., 2017. Extracellular vesicles isolated from the leaf apoplast carry stress-response proteins. Plant Physiol. 173, 728-741.

Rutter, B. D., Innes, R. W., 2018. Extracellular vesicles as key mediators of plant-microbe interactions. Curr. Opin. Plant Biol. 44, 16-22.

Rybak, K., Robatzek, S., 2019. Functions of extracellular vesicles in immunity and virulence. Plant Physiol. 179, 1236-1247.

Samuel, M., Bleackley, M., Anderson, M., Mathivanan, S., 2015. Extracellular vesicles including exosomes in cross kingdom regulation: a viewpoint from plant-fungal interactions. Front. Plant Sci. 6, 766.

Schuster, M., Kilaru, S., Fink, G., Collemare, J., Roger, Y., Steinberg, G., 2011. Kinesin-3 and dynein cooperate in long-range retrograde endosome motility along a non-uniform microtubule array. Mol. Biol. Cell. 22, 3645-3657.

Singh, G., Pratt, G., Yeo, G. W., Moore, M. J., 2015. The clothes make the mRNA: past and present trends in mRNP fashion. Annu. Rev. Biochem. 84, 325-354.

Snetselaar, K. M., Mims, C.W., 1994. Light and electron microscopy of *Ustilago maydis* hyphae in maize. Mycol. Res. 98, 347-355.

Soares, R. P., Xander, P., Costa, A. O., Marcilla, A., Menezes-Neto, A., Del Portillo, H., Witwer, K., Wauben, M., Nolte, T. Hoen E., Olivier, M., Criado, M. F., da Silva, L. L. P., Abdel Baqui, M. M., Schenkman, S., Colli, W., Alves, M. J. M., Ferreira, K. S., Puccia, R., Nejsum, P., Riesbeck, K., Stensballe, A., Hansen, E. P., Jaular, L. M., Ovstebo, R., de la Canal, L., Bergese, P., Pereira-Chioccola, V., Pfaffl, M. W., Fritz, J., Gho, Y. S., Torrecilhas, A. C., 2017. Highlights of the Sao Paulo ISEV workshop on extracellular vesicles in cross-kingdom communication. J. Extracell. Vesicles 6, 1407213.

Statello, L., Maugeri, M., Garre, E., Nawaz, M., Wahlgren, J., Papadimitriou, A., Lundqvist, C., Lindfors, L., Collen, A., Sunnerhagen, P., Ragusa, M., Purrello, M., Di Pietro, C., Tigue, N., Valadi, H., 2018. Identification of RNA-binding proteins in exosomes capable of

 doi:10.20944/preprints201911.0213.v1

interacting with different types of RNA: RBP-facilitated transport of RNAs into exosomes. PLoS One 13, e0195969.

Steinberg, G., 2012. The transport machinery for motility of fungal endosomes. Fungal. Genet. Biol. 49, 675-676.

Steinberg, G., 2014. Endocytosis and early endosome motility in filamentous fungi. Curr. Opin. Microbiol. 20, 10-18.

Stenmark, H., Aasland, R., Driscoll, P. C., 2002. The phosphatidylinositol 3-phosphate-binding FYVE finger. FEBS Lett. 513, 77-84.

Teis, D., Saksena, S., Emr, S. D., 2009. SnapShot: the ESCRT machinery. Cell 137, 182-182 e181.

Toyofuku, M., 2019. Bacterial communication through membrane vesicles. Biosci. Biotechnol. Biochem. 83, 1599-1605.

Vallejo, M. C., Nakayasu, E. S., Matsuo, A. L., Sobreira, T. J., Longo, L. V., Ganiko, L., Almeida, I. C., Puccia, R., 2012. Vesicle and vesicle-free extracellular proteome of Paracoccidioides brasiliensis: comparative analysis with other pathogenic fungi. J. Proteome Res. 11, 1676-1685.

van Niel, G., Charrin, S., Simoes, S., Romao, M., Rochin, L., Saftig, P., Marks, M. S., Rubinstein, E., Raposo, G., 2011. The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. Dev. Cell. 21, 708-721.

van Niel, G., D'Angelo, G., Raposo, G., 2018. Shedding light on the cell biology of extracellular vesicles. Nat. Rev. Mol. Cell Biol. 19, 213-228.

Vargas, G., Rocha, J. D., Oliveira, D. L., Albuquerque, P. C., Frases, S., Santos, S. S., Nosanchuk, J. D., Gomes, A. M., Medeiros, L. C., Miranda, K., Sobreira, T. J., Nakayasu, E. S., Arigi, E. A., Casadevall, A., Guimaraes, A. J., Rodrigues, M. L., Freire-de-Lima, C. G., Almeida, I. C., Nimrichter, L., 2015. Compositional and immunobiological analyses of extracellular vesicles released by *Candida albicans*. Cell Microbiol. 17, 389-407.

 doi:10.20944/preprints201911.0213.v1

Villarroya-Beltri, C., Baixauli, F., Gutierrez-Vazquez, C., Sanchez-Madrid, F., Mittelbrunn, M., 2014. Sorting it out: regulation of exosome loading. Semin. Cancer Biol. 28, 3-13.

Voelz, K., Johnston, S. A., Smith, L. M., Hall, R. A., Idnurm, A., May, R. C., 2014. 'Division of labour' in response to host oxidative burst drives a fatal *Cryptococcus gattii* outbreak. Nat. Commun. 5, 5194.

Vollmeister, E., Schipper, K., Baumann, S., Haag, C., Pohlmann, T., Stock, J., Feldbrügge, M., 2012. Fungal development of the plant pathogen *Ustilago maydis*. FEMS Microbiol. Rev. 36, 59-77.

Walker, L., Sood, P., Lenardon, M. D., Milne, G., Olson, J., Jensen, G., Wolf, J., Casadevall, A., Adler-Moore, J., Gow, N. A. R., 2018. The viscoelastic properties of the fungal cell wall allow traffic of AmBisome as intact liposome vesicles. MBio 9, 02383-02317.

Wang, B., Sun, Y., Song, N., Zhao, M., Liu, R., Feng, H., Wang, X., Kang, Z., 2017. *Puccinia striiformis* f. sp. *tritici* microRNA-like RNA 1 (*Pst*-milR1), an important pathogenicity factor of *Pst*, impairs wheat resistance to *Pst* by suppressing the wheat pathogenesis-related 2 gene. New Phytol. 215, 338-350.

Wang, M., Weiberg, A., Lin, F. M., Thomma, B. P., Huang, H. D., Jin, H., 2016. Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. Nat. Plants 2, 16151.

Weiberg, A., Bellinger, M., Jin, H., 2015. Conversations between kingdoms: small RNAs. Curr. Opin. Biotechnol. 32, 207-215.

Weiberg, A., Wang, M., Lin, F. M., Zhao, H., Zhang, Z., Kaloshian, I., Huang, H. D., Jin, H., 2013. Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. Science 342, 118-123.

Wilson, R. C., Doudna, J. A., 2013. Molecular mechanisms of RNA interference. Annu. Rev. Biophys. 42, 217-239.

 doi:10.20944/preprints201911.0213.v1

Wolf, J. M., Casadevall, A., 2014. Challenges posed by extracellular vesicles from eukaryotic microbes. Curr. Opin. Microbiol. 22, 73-78.

Wolf, J. M., Espadas-Moreno, J., Luque-Garcia, J. L., Casadevall, A., 2014. Interaction of *Cryptococcus neoformans* extracellular vesicles with the cell wall. Eukaryot. Cell 13, 1484-1493.

Xie, J., Kozlov, G., Gehring, K., 2014. The "tale" of poly(A) binding protein: the MLLE domain and PAM2-containing proteins. Biochim. Biophys. Acta 1839, 1062-1068.

Zamith-Miranda, D., Nimrichter, L., Rodrigues, M. L., Nosanchuk, J. D., 2018. Fungal extracellular vesicles: modulating host-pathogen interactions by both the fungus and the host. Microbes Infect. 20, 501-504.

Zander, S., Baumann, S., Weidtkamp-Peters, S., Feldbrügge, M., 2016. Endosomal assembly and transport of heteromeric septin complexes promote septin cytoskeleton formation. J. Cell Sci. 129, 2778-2792.

Zhang, T., Zhao, Y. L., Zhao, J. H., Wang, S., Jin, Y., Chen, Z. Q., Fang, Y. Y., Hua, C. L., Ding, S. W., Guo, H. S., 2016. Cotton plants export microRNAs to inhibit virulence gene expression in a fungal pathogen. Nat. Plants 2, 16153.

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Figures and Figure legends

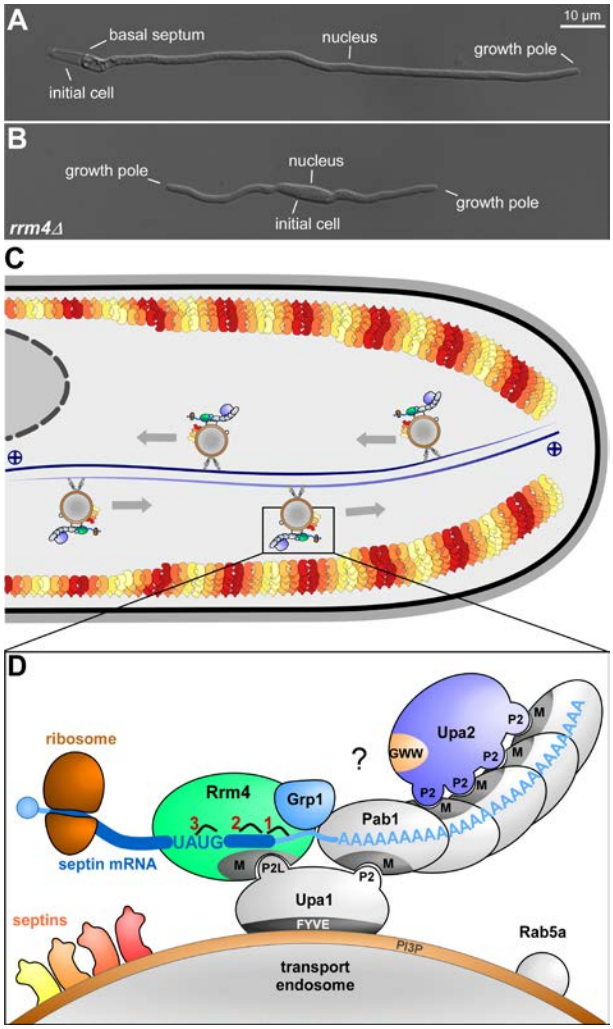

Fig. 1 Endosomal RNA transport machinery in *Ustilago maydis*. (**A**) Unipolar filamentous growth of *U. maydis* laboratory strain AB33, engineered to facilitate genetic studies on filamentous growth (Brachmann et al., 2001). (**B**) Bipolar filamentous growth of *rrm4Δ* strain in AB33 background. Aberrant cell polarity in the absence of the endosome-associated mRNA-binding protein Rrm4 indicates the importance of mRNA transport in polarity maintenance in hyphal cells. (**C**) Model of bi-directional, endosome-associated mRNA transport along microtubules in *U. maydis* hypha. (**D**) Components of the endosomal RNA transport machinery. Rrm4 core mRNA-binding protein and Pab1 poly(A)-binding protein are attached to the surface of Rab5-positive early endosomes via the adaptor protein Upa1. Upa1 is bound to the endosomal surface via a PI3P-binding FYVE domain and possesses PAM2L (P2L) and PAM2 (P2) domains to interact with MLLE domains (M) of Rrm4 and Pab1. Multi-PAM2 protein Upa2 presumably acts as a scaffold for Pab1 proteins on the poly(A) tail of cargo mRNAs, and its GWW motif is important for association of Pab1 on endosomal surface. Rrm4 has three RRM domains (1, 2, 3), which notably bind septin mRNAs, and recognises the UAUG motif via the third RRM domain (3). Additional RNA-binding protein Grp1 co-localises and shares mRNA targets with Rrm4, including septins. Bound mRNAs are translated during transport on endosomes and the translation products are co-transported, as exemplified by shuttling of partially assembled septin hetero-oligomers for increased efficiency.

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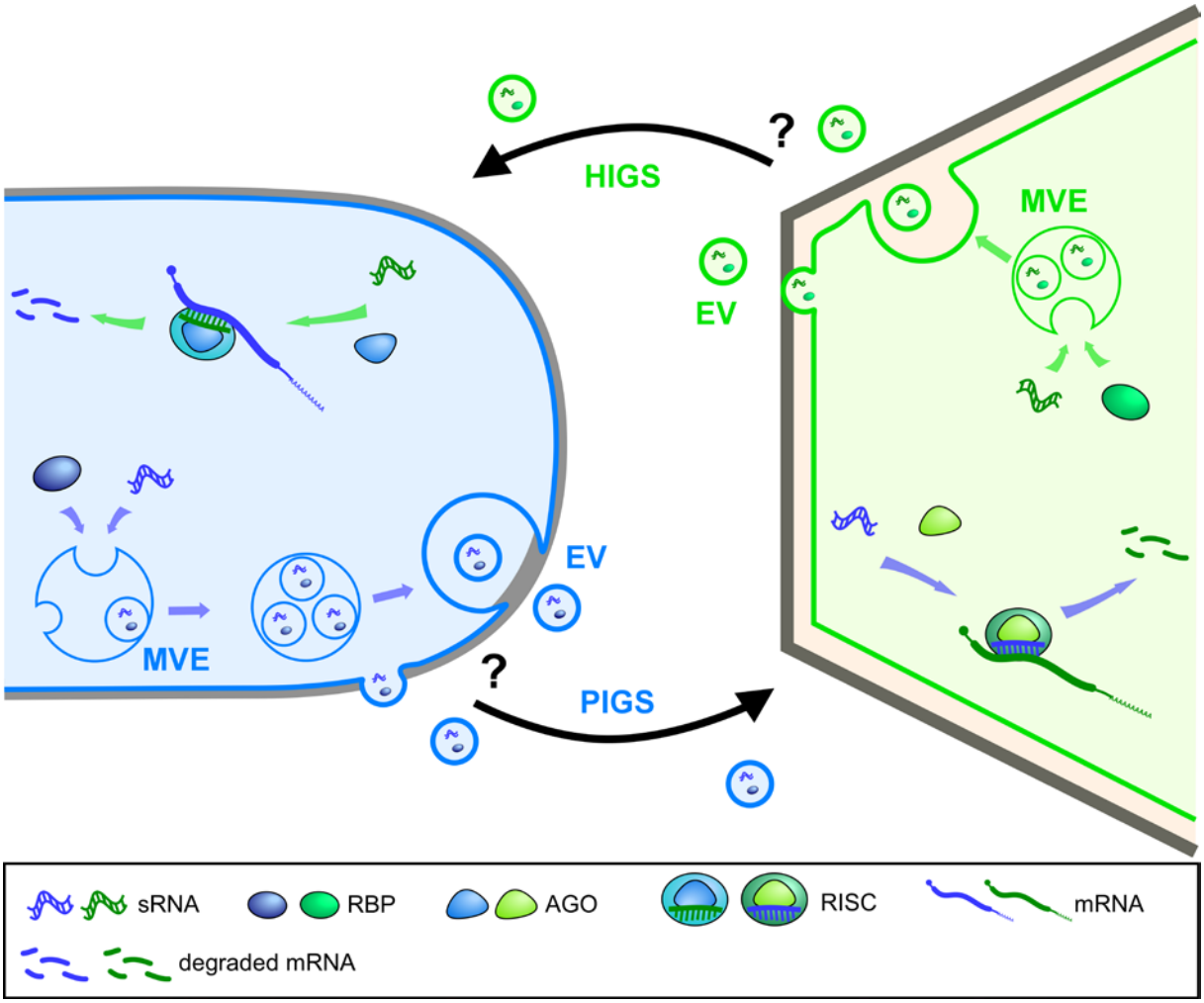

Fig 2 Cross-kingdom RNAi at the fungus-plant interface mediated by extracellular vesicles (EVs). During infection, both the fungus and the plant deploy small RNAs (sRNAs) to silence target genes in the interaction partner, as virulence and defence strategies, respectively. Silencing of fungal pathogen genes by plant host sRNAs is termed host-induced gene silencing (HIGS) and *vice versa*, pathogen-induced gene silencing (PIGS) is brought about by fungal sRNAs in plants. EVs are one of the ways in which sRNAs are transferred between interacting organisms. EVs can be derived from multivesicular endosomes (MVEs) or from budding at the plasma membrane. Endosomes bud inwards during maturation to form intraluminal vesicles, incorporating contents from the cytosol, notably sRNAs and proteins. RNA-binding proteins are thought to be key determinants of RNA loading into EVs. Intraluminal vesicles are released as exosomes upon fusion of the MVE with the plasma membrane. How precisely EVs cross the cell walls and deliver their contents to the recipient cell are currently undetermined.