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.

1. Introduction

The vast majority of fungi grow by forming hyphae. Characteristic for these fungal filaments is a high degree of polarity: they expand at the tip and insert septa in the basal region (Harris, 2019; Riquelme et al., 2018). To achieve this exquisite polar growth, building blocks like lipids, proteins and cell wall material need to be transported efficiently towards the growth pole. Therefore, sophisticated long-distance transport is essential for hyphal growth. Important components are molecular motors that actively transport endosomes and vesicles along microtubules (Riquelme et al., 2018; Steinberg, 2014).

While the exact orchestration of intracellular processes is key for polar growth, intense communication of the growing hyphae with the environment is a second substantial process to guarantee survival. The polar growing cells sense nutrients and utilise them by secreting hydrolytic enzymes from the growing tip. Furthermore, hyphae also exchange information with other organisms like microbes, plants and animals. A well-studied example is the role of fungi as pathogens. Evidence is accumulating that also in this case membranous carriers like extracellular vesicles are crucial delivery vehicles. Intriguingly, for intra- as well as for intercellular transport, a functionally important links between membranous transport vehicles and RNA trafficking have been disclosed.

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) play important roles in escorting and transporting cargo RNAs and thus contain designated domains to interact with specific elements in their targets. 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 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 mechanisms 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 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 (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 (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).

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 analysis mapped sequences bound by Rrm4 with single-nucleotide resolution. This showed groups of transcripts

with different patterns of binding including precisely the start or stop codons, the ORF, and most prominently, the 3´ untranslated region (UTR) (Fig. 1C-D; Olgeiser et al., 2019). Differential binding specificities of the three RRM domains, in combination with other protein interactors, might bring about different binding patterns on the target mRNA. Supporting this notion, the third RRM domain recognizes the sequence motif UAUG. Furthermore, the small glycine-rich RNA-binding protein Grp1 was found to share targets with Rrm4, particularly in 3´ UTRs (Fig. 1C-D; 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, such as filaments, with a gradient emanating from the hyphal growth pole (Fig. 1C-D; 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, a 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. 1C-D).

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 interacts with Rrm4 but unexpectedly, its PAM2 motif was dispensable for this function (Pohlmann et al., 2015). However, it was found to contain two PAM2-like sequences (PAM2L), which mediate the 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. 1D; 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. 1D; Jankowski et al., 2019).

To learn more about the identity of the transport endosomes, the conserved factor Did2 was studied. It 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 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 its conservation across Basidiomycota and the distantly related Mucoromycota (Müller et al., 2019). Endosomal shuttling of the heterologously expressed Rrm4 orthologue from fungi as distant as *Rhizophagus irregularis* in *U. maydis*, suggests a high degree of functional conservation (Müller et al., 2019). By contrast,

potential homologues of components of the core endosomal transport machinery like Rrm4, Upa1 and Upa2 were not found in Ascomycota. However, microtubule-dependent shuttling of the RNA-binding protein Gul1 was recently reported in hyphae of the ascomycete *Neurospora crassa* (Herold et al., 2019). Hence, long-distance transport of mRNAs along microtubules might be operational also in ascomycetes, however the core machinery appears to be different.

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 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 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). Comparative proteomics of fungal EVs displayed not only high diversity, but also revealed core sets of cargo proteins, indicating some degree of conservation 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). Of note, 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, a number of 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.

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. Noteworthy, both the EV protein and RNA cargoes are essential for this effect. Proliferation of the non-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 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). Pathogen-induced gene silencing (PIGS; Fig. 2) by a fungal pathogen was initially discovered as a virulence strategy for *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.

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-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

In this review article, we describe how two major membrane-associated RNA transport mechanisms - endosomal and EV-mediated RNA trafficking - function in fungi. Although these two modes of transport seem to lack an obvious connection, there are most likely linked at the subcellular level by sharing components of the endosomal pathway from which they origin. In Table 1, we compare key features of intracellular and extracellular RNA transport.

Table 1: Comparison of intra- and extracellular membrane-associated RNA transport.

	Intracellular RNA transport	**Extracellular RNA transport**
Organelle	Rab5-positive early endosomes	Extracellular vesicles (EVs)
Origin	Endocytic pathway, ESCRT	Endocytic pathway, ESCRT, MVEs
Function	Spatiotemporal regulation of mRNA translation during polar growth	Cross kingdom RNAi, intercellular communication

RNA-binding proteins	Rrm4, Grp1, Pab1 (*U. maydis*) Gul1 (*N. crassa*)	Several uncharacterized RBPs found in EVs with currently unknown function in RNA transport
Cargo RNAs	mRNAs	Small regulatory RNAs, tRNA halves, other types of RNAs

During endocytosis, endosomes mature by forming MVEs to target protein cargo for degradation or for recycling towards the plasma membrane (Huotari and Helenius, 2011). However, early endosomes also function as multipurpose platforms in long-distance intracellular mRNA transport. They deliver mRNAs and cognate translation products for spatio-temporal regulation of protein expression within fungal hyphae, for example during polar growth (Baumann et al., 2014). Interestingly, also in the case of extracellular RNA transport, the endocytic formation of MVEs starting with maturing early endosomes has been proposed to be one important pathway that leads to the formation of EVs (Fig 2).

We envision that key factors of both intra- and extracellular RNA transport are RBPs as they constitute key regulatory elements for RNA selection, stabilisation and transport. They form higher-order RNPs and link these transport units physically to membranes, for example, by direct interaction of RBPs with specific lipid structures. Alternatively, adaptor proteins, such as Upa1, connect cargo RNPs with transport membranes via lipid-binding domains (Pohlmann et al., 2015). Loading of MVEs with cargo RNAs for extracellular transport is likely mediated by transport RBPs, too, although their identities are not known in fungi, yet (Table 1). Hence, studying and comparing these seemingly different RNA transport processes is very informative and highly rewarding to understand the various facets of RNA and membrane trafficking. 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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Figures and Figure legends
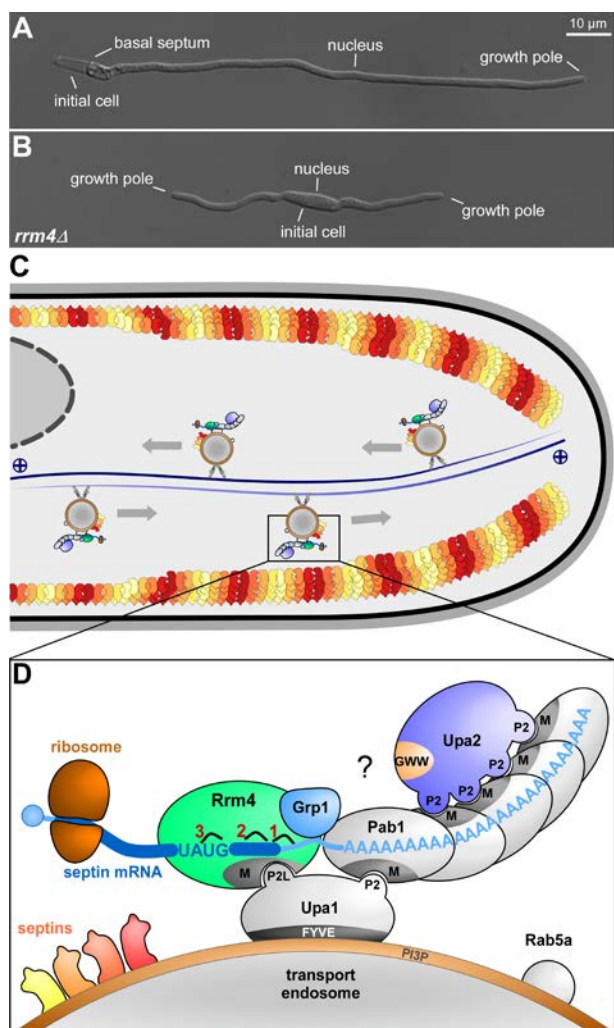
Fig. 1 Endosomal RNA transport machinery in *Ustilago maydis*. (**A**) Unipolar filamentous growth of *U. maydis* laboratory strain AB33, engineered to facilitate genetic studies on filamentous growth (Brachmann et al., 2001). (**B**) Bipolar filamentous growth of *rrm4Δ* strain in AB33 background. Aberrant 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 (growth pole at the right, nucleus in grey). Depicted are cortical septin filaments formed by septin heterooctamers (yellow, orange and red subunits). An array of antiparallel microtubules with shuttling endosomes is drawn in the center (plus ends [+] of microtubules are indicated). The plus-end directed motor Kin3 is given as an example. Local translation of septin mRNAs on the cytoplasmic surface of endosomes results the assembly of heteromeric septin complexes on endosomes. These heteromeric protein complexes are transported towards the growth pole to enable efficient formation of septin filaments forming a gradient emanating from the hyphal tip. The cytoplasmic surface of transport endosomes in enlarged in D. (**D**) Components of the endosomal RNA transport machinery. Rrm4 core mRNA-binding protein (green oval) and Pab1 poly(A)-binding protein (grey oval) are attached to the surface of Rab5a-positive transport 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 (blue oval) presumably acts as a scaffold for Pab1 proteins on the poly(A) tail of cargo mRNAs, and its GWW motif (orange patch) is important for association of Pab1 on endosomal surface. The interaction partner of the GWW motif is currently unknown as indicated by the question mark. 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 (light blue oval) 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.
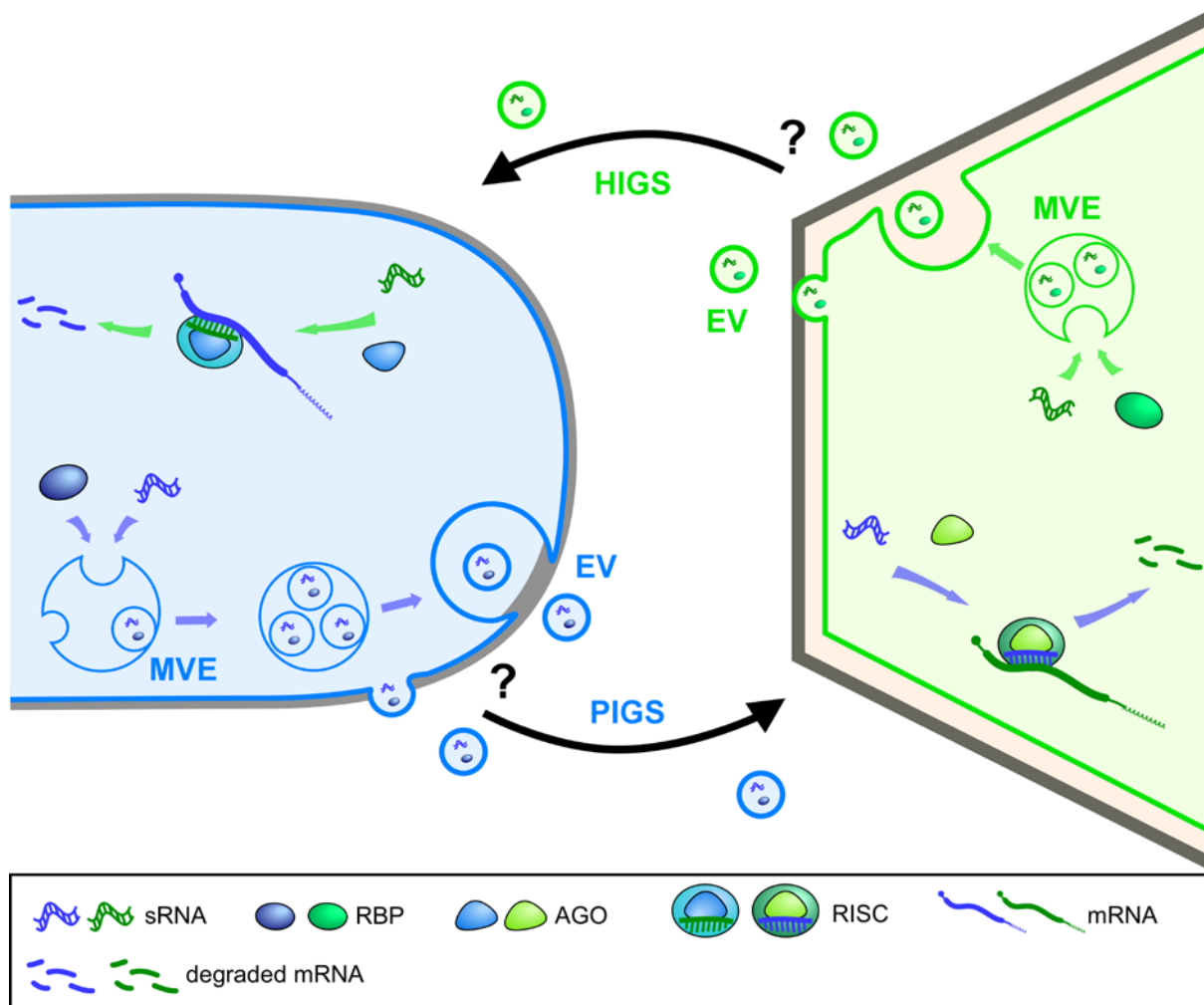
Fig 2 Cross-kingdom RNAi at the fungus-plant interface mediated by extracellular vesicles (EVs). During infection, both the fungus and the plant deploy small RNAs (sRNAs) to silence target genes in the interaction partner, as virulence and defence strategies, respectively. Silencing of fungal pathogen 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.