Cargo sorting into, and the interactive effects of, membrane vesicles: knowledge pool and gaps in fungal phytopathogens

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September 14, 2023

Abstract

Organisms from all kingdoms of life release membrane vesicles, which are tiny and spherical structures made of a lipid bilayer. Membrane vesicles carry out a number of functions, such as forming new cell membranes, removing waste products from the cell, and transporting lipids and other substances from parent to recipient cells. The payloads often contained in the vesicles are sorted via the endosomal sorting complex required for transport (ESCRT) pathway in a stepwise manner. Alterations to this endomembrane system reduces formation of vesicles and aberrant endosomal compartments. Furthermore, in pathogenic fungi, the deletion of ESCRT genes negatively effects virulence and growth, suggesting the ESCRT pathway has links to disease. However, only a few fungal species have to date been evaluated for the ESCRT pathway. In this review, we evaluate recent developments in the ESCRT pathway of fungi that infect plant hosts and its role in pathogenesis. This will provide an overview of EV-mediated cell-cell communication during host-pathogen interactions.

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Abstract

Organisms from all kingdoms of life release membrane vesicles, which are tiny and spherical structures made of a lipid bilayer. Membrane vesicles carry out a number of functions, such as forming new cell membranes, removing waste products from the cell, and transporting lipids and other substances from parent to recipient cells. The payloads often contained in the vesicles are sorted via the endosomal sorting complex required for transport (ESCRT) pathway in a stepwise manner. Alterations to this endomembrane system reduces formation of vesicles and aberrant endosomal compartments. Furthermore, in pathogenic fungi, the deletion of ESCRT genes negatively effects virulence and growth, suggesting the ESCRT pathway has links to disease. However, only a few fungal species have to date been evaluated for the ESCRT pathway. In this review, we evaluate recent developments in the ESCRT pathway of fungi that infect plant hosts and its role in pathogenesis. This will provide an overview of EV-mediated cell-cell communication during host-pathogen interactions.

Key words: Plant pathogens, fungi, endocytosis, ESCRT pathway, extracellular vesicles, ubiquitination

Introduction

Extracellular vesicles (EVs) mediate cell-cell-dependent environmental responses (Rodrigues et al., 2015; Anand et al., 2019, Baldrich et al., 2019). Numerous studies have isolated and characterized fungal pathogen EVs and found that they produce phytotoxic effects, among other things, when inoculated on plant tissues such as leaves (Bleackley et al., 2020). This suggests that cargo within these vesicles can be disease-promoting. How this cargo is sorted is currently elusive in plant pathogenic fungi, which limits our understanding of virulence factors transmitted through non-canonical pathways. A repertoire of EV constituents, including nucleic acids, proteins, and secondary metabolites often reflect the pathophysiological state of the cell from which EVs are secreted. These cargos can be taken up by other cells naked or enclosed in the EV membrane to yield the different EV-mediated physiological states in the acceptor cells (Koch et al., 2020). As EVs carry various bioactive molecules, many of which facilitate cell-cell communication (Rodrigues et al., 2015; Anand et al., 2019), they have also been implicated in plant-fungal interaction and pathogenesis. For instance, EVs of several species of fungi have been shown to traffic virulence factors, such as cell wall degrading enzymes, protein effectors, and toxins with phytotoxic effects on their plant host tissues (Bleackley et al., 2020; Costa et al., 2021; Regente et al., 2017). However, there is still a lack of understanding about the regulatory mechanisms involved in cargo sorting, packaging and trafficking of the respective vesicles, especially in filamentous fungi.

The ESCRT (*e* ndosomal *s* orting *c* omplex*r* equired for *t* ransport) pathway is utilized by many eukaryotes including fungi whereby it is involved in the sorting and trafficking of molecules within the endosomal system. This system is involved in the formation and sorting of endosomal vesicles, which are part of the endocytic pathway responsible for internalizing molecules from the cell surface and delivering them to specific destinations within the cell. In filamentous fungi, the ESCRT pathway plays a role in the biogenesis of filamentous growth and pathogenesis (Zheng et al., 2018; Sun et al., 2022). Studies have shown that the ESCRT pathway is required for the conventional organization of the fungal cytoskeleton (An et al., 2006; Henne et al., 2013), which is essential for the formation of hyphae. The ESCRT pathway also plays a role in the splitting of daughter nuclei during cell division and the sorting of proteins destined for the plasma membrane (Henne et al., 2013). Additionally, the ESCRT pathway is involved in the formation of endosomal vesicles that are important for nutrient uptake and stress response (Fan et al., 2015; Wang et al., 2020). In pathogenic fungi, the ESCRT pathway also plays a role in the secretion of virulence factors and evasion of host immunity (Regente et al., 2017; Martínez-López et al., 2022).

The ESCRT pathway is vital for endocytosis whereby it allows for the packaging of extracellular materials and

membrane proteins into EVs for trafficking into the cytoplasm (Fig 1**(A)** , Ahmed et al., 2019). Endocytosis is characterized by the presence of late endosomes or multivesicular bodies (MVBs), which harbour intraluminal vesicles (ILVs) that usually arise from the invagination and budding of the endosomal membrane (Haag et al., 2015; Ahmed et al., 2019). The process of endocytosis is fundamental to various cellular process ranging from signal transduction to morphogenesis (Schimid et al., 2014). In certain fungal species, endocytosis also plays a role during interaction with plants including in the apical growth of hyphae (Toshima et al., 2006; Bielska et al., 2014). In addition to growth and development, ESRCT-based regulation of cellular function also appears to be crucial for adaptation and response to both external and internal stimuli such as biotic and abiotic stress factors (Mosesso et al., 2019; Rosa et al., 2020). Consequently, many of the basic components of the ESCRT pathway are conserved across eukaryotes, albeit with notable lineage-specific adaptations in some taxa (Leung et al., 20008).

Most of the significant contributions to our understanding of ESCRT derive strongly from studies on model organisms (Thompson et al., 2005; Vaccari & Bilder, 2005; Herz et al., 2006; Spitzer et al., 2006). Adding to these are several studies highlighting the key roles of ESCRT proteins in mammals (Pornillos et al., 2002; Xu et al., 2003; Skibinski et al., 2005; Parkinson et al., 2006; Saksena and Emr, 2009; Stuffers et al., 2009; Hurley et al., 2015). The ESRCT pathway was originally discovered in Archaeal cytokinesis (Lindas et al., 2008; Samson et al., 2008) and in fungi it was first discovered in the model fungus*Saccharomyces cerevisiae* (Emr et al., 2001). In this yeast, the ESCRT pathway's constituents were named based on their functions in sorting ubiquitinated membrane proteins into lysosome/vacuole lumens for degradation (Katzmann et al., 2001; Babst et al., 2002a; Xie et al., 2019b). The pathway represents a complex endomembrane system that consists of five complexes, namely ESCRT-0, -I, -II -III and Vps4 (*v* acuolar *p* rotein *s* orting *4*). Together with various accessory proteins, the individual elements of the pathway act in concert to form MVBs during diverse processes including cytokinesis, membrane repair and autophagy (Roxrud et al., 2010; Henne et al., 2013; Hurley et al., 2015).

As in other eukaryotes, fungal MVBs are critical for transporting ubiquitinated membrane proteins to the vacuole with the aid of the ESCRT machine, which both recognizes and packages these ubiquitin-modified proteins into the ILVs contained inside MVBs (An et al., 2006; Hurley and Hanson, 2010; Henne et al., 2011). Studies in yeast also showed that ILVs form when MVB membranes evaginate and undergo fission (Ahmed et al., 2019; Anand et al., 2019). Others have reported that impairment in the ESCRT apparatus can lead to reduced formation of ILVs and aberrant endosomal compartments (Raymond et al., 1992; Xie et al, 2019a). In the filamentous fungal pathogen, *Fusarium graminearum* , such defects can significantly impact cellular processes like deoxynivalenol production, growth and pathogenicity, as well as sexual and asexual reproduction (Xie et al. 2019a). However, details regarding this pathway have been considered in only a few phytopathogens, most notably*Magnaporthe oryzae* and *F. graminearum* (Oh et al., 2012; Xie et al., 2016; Cheng et al., 2018; Xie et al., 2019a, b; Que et al., 2019). Currently, most fungal work pertaining to the ESCRT pathway is focused on human pathogenic yeasts including *Cryptococcus neoformans* and *Candida albicans* (Godinho et al., 2014; Hu et al., 2015; Zhang et al., 2015; Park et al., 2020). The elucidation of the core functions of the ESCRT pathway in fungal plant pathogens represents a critical aspect given the functional importance of this pathway. Therefore, this review reflects on the recent ESCRT discoveries as they relate to filamentous fungi and touch on the relevance of released EVs, which are the end-products of ESCRT, in fungal biology and fungal-host interactions.

*V*acuolar *p*rotein *s*orting genes (Vps) facilitate the assembly of vacuoles

Fungal vacuoles are complex cellular organelles involved in several functions including homeostatic regulation and degradative processes among others (Klionsky et al. 1990). During EV biogenesis, fusion of MVBs occurs with vacuoles and autophagosomes and the MVB-endocytosed cargo is delivered and then undergo degradation as means of cellular homeostasis (Fig 1 **(D)** , Luzio et al., 2003; Luzio et al., 2007). Induced autophagy has been shown to result in reduced EV release due to increased fusion of the MVBs to the autophagosomes (Fader et al., 2008). Though little is known about directive mechanisms involved in trafficking of MVBs to either the plasma membrane or the vacuole, these organelles serve an important role in maintaining cytosolic

balance.

Vacuolar proteins are recognized by certain cellular components and are sorted, packaged into transport vesicles, and delivered to the vacuole through a prevacuolar compartment (Hedman et al., 2007). This process involves the assembly of the Vps genes (e.g., Vps 23 or Vps 27) into the five sub-complexes of the pathway, which in a stepwise '*modus operandi* ,' interact, recognize and sort ubiquitinated cargo (Fig 2, Mosesso et al., 2019). These proteins are encoded by class E vacuolar protein sorting genes that are structurally classified as type A-F. Among these, the first Vps genes were classified into type A, B and C, with class D, E, and F added later (Hedman et al., 2007). These Vps genes are associated with the formation of the vacuole. For instance, mutants of the class A genes exhibit defects in acidification of the vacuole, class B mutants exhibit small-vacuole like organelles, which together with class F mutants are characterized by large to fragmented small vacuole-like structures (Banta et al., 1988; Hedman et al., 2007).

Class C Vps is associated with normal assembly of the vacuole as mutants display severely aberrant vacuole formation and sensitivity to osmotic stress in *S. cerevisiae* (Banta et al., 1988). Although their absence has minimal effects in vacuolar biogenesis, and trafficking of endocytosed proteins, class D gene products are limited to the vesicular pathway (Bryant et al., 1998). Class E Vps are important in forming unique class E compartments that house the vacuolar hydrolase carboxypeptidase S (CPS), a transmembrane protein required in the vacuole (Raymond et al., 1992; Shaw et al., 2001; Coonrod et al., 2010). While CPS accumulates in class E compartments, CPS that fails to reach the vacuole lumen remains in the limited membranes of the vacuole (Reggiori et al., 2001; Bowers et al., 2005; Piper et al., 2007). In yeast, about 15 components of the class E Vps family, most of which associated with MVB sorting and augmented endosomal compartments, were reported (Katzmann et al., 2001).

It has also been demonstrated in yeasts and mammals that Vps proteins interact sequentially. This results in the development of various cellular events such as scission, transport, and the formation of distinct budding membranes (Hurley and Hanson, 2010; Henne et al., 2011; Schuh and Audhya, 2014; Tang et al., 2016). Hence, ESCRT-I and ESCRT-II have been identified as crucial elements in trafficking ubiquitinated cargo to the vacuole. ESCRT- 0 on the other hand, is not as essential in either mammals or yeast for the aforementioned functions, which is in accordance with the dynamic endosomal pathway in plants where ESCRT- 0 is also absent (Henne et al., 2011; Richardson et al., 2011; Hurley, 2015).

Components and assembly of the ESCRT-dependent pathway in fungi

The ESCRT-0 heterodimer is comprised of two subunits, Vps27 and Hse1 (Fig 2), found in *S . cerevisiae* (Williams and Urbé, 2007). They are orthologous to the human protein Hrs (*h* epatocyte growth factor *r* egulated tyrosine kinase *s* ubstrate) and STAM (*s* ignal *t* ransducing *a* daptor *m* olecule), respectively (Raiborg and Stenmark, 2009; Henne et al., 2011; Mosesso et al., 2019; Xie et al., 2019a). The ubiquitin-interacting motifs (UIMs) of both subunits comprise of an *N* -terminal VHS (named after the proteins *V* ps27, *H* rs and *S* TAM) domain (Ren and Hurley, 2010). Although both subunits display some structural resemblance, the Vps27/Hrs structure is slightly different in that it encompasses a FYVE (named after the proteins *F* ab1, *Y* OTB,*V* ac1 and *E* EA1) zinc finger domain (Katzmann et al., 2003; Henne et al., 2011). The cysteine-rich FYVE binds to an endosomal lipid, phosphatidylinositol (Ptdlns) 3-phosphate (PI3P), that is crucial for MVB formation and endocytic trafficking and required for the Vps27/Hse1 complex functions and localization (Gillooly et al., 2001; Raiborg et al., 2003; Katzmann et al., 2003; Xie et al., 2016). In*Drosophila melanogaster* , inhibition of PI3P alters the recruitment of Hrs and consequently obstruct MVB formation (Lloyd et al., 2002; Raiborg et al., 2003). Moreover, defects in the Vps27 UIM do not impact vesicle budding and delivery onto the MVB lumen, but hinder sorting of ubiquitinated cargo protein into MVBs (Katzmann et al., 2001, 2002; Piper and Katzmann, 2007; Conibear, 2010).

The ESCRT-I complex is comprised of four subunits, namely Mvb12, Vps23, Vps37 and Vps28 (Fig 2). However, only Vps23 and Vps28 (Table 1) are present in *F. graminearum* and interact with each other (Xie et al., 2019a). Vps23 and Vps28 also interact with both ESCRT-0 and ESCRT-II at opposite ends of the complexes (Katzmann et al., 2001; Chu et al., 2006; Curtiss et al., 2007; Henne et al., 2011). In mammals,

there are multiple isoforms of these ESCRT-I subunits, which may mirror their variations based on tissue-specificity (Henne et al., 2011). The*N* -terminal ubiquitin E2 variant (UEV) domain of the Vps23 subunit propels to the ESCRT-I stalk, while the UEV binds to the PTAP-like motifs (*P* ro-*T* hr/Ser-*A* la-*P* ro) of Vps27, an upstream MVB sorting component, then recruits the ESCRT complex protein to the endosomal membrane (Katzmann et al., 2003; Xie et al., 2019a).

The ESCRT-II protein sub-complex is comprised of three subunits, namely Vps22, Vps36, and Vps25 (Babst et al., 2002b). Through the interaction between Vps25 and Vps20, ESCRT-II recruits downstream ESCRT-III, thus, in turn activating this protein sub-complex (Xie et al., 2019a). The interaction of ESCRT-II with ESCRT-I involves provision of the endosomal localization, the GLUE (*G* RAM-*l* ike *u* biquitin-binding in *E* ap45) domain of Vps36 and Vps28 subunits that bind to ubiquitin PI3P, while Vps25 binds Vps20 and attach to ESCRT-III, thus exhibiting the crucial role of ESCRT-II in activating the formation of ESCRT-III complex (Teo et al., 2006; Hanson et al., 2009; Henne et al., 2011).

The ESCRT-III sub-complex is comprised of four major subunits, Vps20, Snf7/Vps32, Vps24, and Vps2 (Fig 2). They occur as monomers in the cytosol and cluster rapidly on the endosomal membrane into an active complex (Babst et al., 2002a; Xie et al., 2019b). SnF7 homo-oligomerise post interaction with Vps20 and it is also the most abundant component of ESCRT-III and can self-interact as reported in previous studies (Lin et al., 2005; Teis et al., 2008; Xie et al., 2019b). The major subunits may contain a few accessory or adaptor proteins such as Did2, Bro1, and Vps60 (Table 1, Ahmed et al., 2019). ESCRT-III protein sub-complex is unique from the first three ESCRT complexes because it does not form part of the stable cytoplasmic complex but rather exist in a closed autoinhibited condition in the cytoplasm (Henne et al., 2011; Xie et al., 2019b).

According to Teo et al., (2004), ESCRT-III is triggered by the interaction between Vps25 and Vps20 from which it forms a complex, as it is recruited to the endosome. Prior to the activation of ESCRT-III, ESCRT-I and ESCRT-II complexes link through the interactions between Vps23 and Vps22, Vps23, and Vps36, and finally Vps28 and Vps36, during which the subunits Vps25 and Vps36 of ESCRT-II complex directly interact with ESRCT-III protein complex (Xie et al., 2019b). Despite the differences in the components of some ESCRT complexes, the sequential recruitment and clustering of the ESCRT complexes from interactions among ESCRT protein subunits in *F. graminearum* has exhibited some consistencies comparable to the yeast and mammalian models (Xie et al., 2019b). This suggests that the main interactions of this pathway are well-conserved in eukaryotes (Martin-serrano et al., 2003; Von Schwedler et al., 2003; Bowers et al., 2004; Teis et al., 2008; Xie et al., 2019b).

Lastly, ESCRT-IV disassembles the ESCRT-III complex when ESCRT-II interacts with Vps20, which recruits SnF7 (Tang et al., 2016). SnF7 has been found to play roles that are vital to the pathogenic process in fungi including cell wall integrity, endocytosis, vesicle trafficking, as well as growth and differentiation (Table 1) (Cheng et al., 2018). SnF7 then recruits Vps24 and Vps2, thus completing the assembly of ESCRT-III, shortly after Vps2 engages Vps4 (Obita et al., 2007; Teis et al., 2008; Henne at al., 2011). For stabilization, SnF7 may also recruit adaptor proteins from ESCRT-III i.e., Bro1 and Doa4 DUBs (Odorizzi et al., 2003; Luhtala and Odorizzi, 2004).

For ESCRT-III to disassemble from the membrane, energy is required and that is usually provided by class I AAA (*A* TPase *a* ssociated with various cellular *a* ctivities) ATPase Vps4 (Fig 1) (Babst et al., 1998). As with SnF7, other ESCRT-III accessory proteins also regulate the interaction between ESCRT-III and Vps4 (i.e., Did2, Vta1 or Vps60) to modulate the functions of Vps4 in several ways, such as facilitating the self-interaction of Vps4, Vps4 interaction with ESCRT-III subunits, or the stimulation of ATP hydrolysis (Ahmed et al., 2019). Furthermore, Vta1 directly interacts with Vps60 of the ESCRT-III complex. A super complex that is stabilized by a flexible Vta1 cap is formed when Vta1 stimulates the ATPase activity of Vps4 (Ahmed et al., 2019).

Posttranslational modification of protein cargo via ubiquitination

Ubiquitin is a highly conserved regulatory protein, consisting of 76 amino acid residues (Wang et al., 2012;

Ahmed et al., 2019; Wang et al., 2019). Ubiquitination is a process in which a ubiquitin is covalently attached to a targeted lysine residue on the cytoplasmic tail of a transmembrane protein (Wang et al., 2012; Ahmed et al., 2019). Monoubiquitination serves as a signal for the lysosomal targeting process, where monoubiquitinated proteins are trafficked to the lysosome (Raiborg et al., 2003; Anand et al., 2019). By contrast, polyubiquitinated proteins are intended for proteolysis, (Hicke et al., 2001). Ubiquitination thus appears to serve centrally both in endocytosis and protein turnover (Leung et al., 2008; Mosesso et al., 2019).

The process of ubiquitination (and de-ubiquitination) takes place along the endosomal membrane. Ubiquitination involves the activities of three enzymes, namely ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), which are used for the development of iso-peptide bonds between the C-terminal glycine of ubiquitin and the amine of the lysine (K) (e.g., -63 etc.) residue of the target protein (Bhoj and Chen, 2009; Liu and Xue, 2011; Schwihla and Korbei, 2020).

The E3 ligase mostly regulates the specificity of the targeted protein (Oh et al., 2012). So far, only seven lysine residues within the ubiquitin protein are used in the ubiquitination reaction to create ubiquitination chains (i.e., K6, -11, -27, -29, -33, -48, and -63) and may lead to mono-ubiquitinated or poly-ubiquitinated substrates (Wang et al., 2012; Schwihla and Korbei, 2020). The most common polyubiquitin chains are, however, K48- and K63-linked polyubiquitin chains, with K63 reportedly associated with endocytic transportation (Rodrigo-Brenni et al., 2010, Paez Valencia et al., 2016). In yeast, monoubiquitination alone is sufficient to stimulate endocytic internalization of ubiquitinated cargo into MVBs (Lucero et al., 2000; Kim et al., 2007). However, the existence of K63-linked chains also proved to be particularly important during the MVB sorting of CPS (Lucero et al., 2000; Erpapazoglou et al., 2008; Kim et al., 2009; Lauwers et al., 2010). Similarly, this phenomenon has been reported in*Arabidopsis* where K63 is the second most abundant polyubiquitin chain after K48 (Kasai et al., 2011; Lu et al., 2011; Leitner et al., 2012; Martins et al., 2015; Dubeaux et al., 2018). Furthermore, defects in the polyubiquitin K63 link chains from *M. oryzae* are alluded to cause substantial alteration on fungal growth and development as well as morphological changes (Oh et al., 2012).

Sorting of ubiquitinated-protein cargo into ILVs

Ubiquitination plays major roles in trafficking plasma membrane proteins into the endosomal system (Schwihla and Korbei, 2020). Endosomal ubiquitin modification is critical for ESCRT recognition and sorting of ubiquitin-modified protein cargo into ILVs (Williams and Urbé, 2007; Villarroya-Beltri et al., 2014). This endosomal modification facilitates ubiquitinated proteins to lysosomes through MVBs (Williams and Urbé, 2007). Ubiquitination also serves as essential signal for proteins lacking signal peptides recruited to the ESCRT-dependent pathway during MVB sorting (Piper and Luzio, 2001; Agrawal et al., 2010)

Early endosomes, which originate from the *trans* -Golgi network (TGN) as part of the endocytic membrane transport pathway, are major sorting stations that accept molecules from the extracellular environment. On the other hand, MVBs are prelysosomal organelles acting during endocytosis to regulate incoming and outgoing traffic through homotypic and heterotypic fusion events (Luzio et al., 2003; Luzio et al., 2007; Huotari and Helenius, 2011). Although, homotypic fusion of MVBs occurs with lysosomes and autophagosomes, heterotypic fusion is different in that it can occur with the plasma membrane. But in both cases, fusion results in the delivery of endocytosed cargo. In the case where MVBs fuse with the plasma membrane, ILVs are released into the extracellular space as exosomes, and this has been implicated in cell-cell and *trans* -kingdom communication including plant-fungal interaction (Cai et al., 2018).

The sorting of MVB is important for a series of biological events, including endosomal sorting, organelle biogenesis, and vesicular transportation (Colombo et al., 2014; Yáñez-Mó et al., 2015; Hyka et al., 2017). Assembly of these molecular cargos takes place inside membrane microdomains of the MVBs and on the plasma membrane (Kuipers et al., 2018). Several ESCRT proteins have been implicated as constituents enriched in EVs, particularly 30-150 nm cup-shaped vesicles (i.e., Tsg101, a protein associated with ESCRT-I, and Alix, an accessory protein associated with ESRCT-III, both of which make up components of the EVs) and are frequently used as biomarkers for them (Juan and Fürthauer, 2018; Anand et al., 2019).

Prior to being sorted into ILVs, protein cargo transverse between membrane-bound protein complexes that

recognize and bind to the ubiquitin chains attached to them (Paez Valencia et al., 2016). Equipped with the ubiquitin-interacting domains essential for cargo sorting, the ESCRT complexes -0, -I and -II interact stringently with ubiquitinated cargos and traffic them to the endosomal MVB pathway (Fig 3**(B)**). These sub-complexes can recognize and retain endosomal ubiquitinated cargos, and transport them into MVBs (Anand et al., 2019; Mossesso et al., 2019). In addition, endocytosis modulators such as epsins (e.g., Epsin15) possess ubiquitin-interacting motifs (UIM) that function in ubiquitin recognition (Mayers and Audhya, 2012). The yeast homologs of epsin, Ent1 and Ent2, are reportedly essential for endocytosis of ubiquitin-dependent α-factor receptor that requires ESCRT-0 Vps27 for sorting (Shih et al., 2002; Raiborg et al., 2003). Finally, membrane scission of the ECRT complex is promoted by the recruitment of the ESCRT-III assembly together with its accessory proteins including Bro1 or Vps60 (Moreno-Gonzalo et al, 2014; Stoorvogel, 2015; Fig 2).

Ubiquitin detachment (de-ubiquitination) and protein cargo packaging

The process of ubiquitination can be reversed or eliminated by de-ubiquitination enzymes (DUBs). These enzymes represent a large family of proteases that are responsible for cleaving ubiquitin from proteins (Schwihla and Korbei, 2020). In fungi and mammals, deubiquitination appears to neutralize and maintain the amount of ubiquitinated membrane proteins trafficked for degradation through recycling cargo back onto the plasma membrane (McCullough et al., 2004; Schwihla and Korbei, 2020). De-ubiquitination also represents a crucial step during the formation of MVBs.

As MVBs form, prior to incorporation of the endocytic cargo into ILVs, DUBs remove their attached ubiquitin (Raiborg et al., 2009). For instance, the ESCRT-III Snf7 attached to Bro1 activates de-ubiquitination by recruiting Doa4 (*d* egradation *o* f*a* lpha-*4*) to the endosome before incorporation of ubiquitin modified cargos into MVBs (Amerik et al., 2000b; Katzmann et al., 2001; Odorizzi et al., 2003; Luhtala and Odorizzi 2004; Wemmer et al., 2011). However, the de-ubiquitination process does not preclude certain ubiquitinated proteins from remaining in the ILVs. It has been documented that certain EV constituents contained polyubiquitinated non-integral membrane proteins (Buschow et al., 2005). Though this phenomenon is not entirely understood, ubiquitinated proteins in EVs are believed to may have escaped de-ubiquitination and, as such, they may indicate microautophagy uptake or the cytoplasmic cargo destined to degradation in lysosomes (Buschow et al., 2005).

DUBs actively link with the ESCRT components (e.g., SnF7-Bro1) to modify and control the status and fate of ubiquitinated cargo (Wright et al., 2011; Que et al., 2019). The ESCRT-III sub-complex does not have a ubiquitin recognizing domain, yet it can actively recruit DUBs such as Ubp7 (*ub* iquitin-specific processing *p* rotease *7*) and Doa4 that are regulated by ESCRT-III adaptor protein Bro1 in yeasts (Williams and Urbé, 2007; Roxrud et al., 2010; Que et al., 2020). In*S* . *cerevisiae* Doa4 plays a major role in ubiquitin homeostasis and ubiquitin-dependent proteolysis (Amerik et al., 2000b; Que et al., 2020). In addition, Doa4 apparently also recovers ubiquitin from ubiquitinated cargo en-route to MVBs (Que et al., 2020). Nevertheless, biological functions of most DUBs are not known in pathogenic plant pathogenic fungi (Wang et al., 2018; Que et al., 2020).

ESCRT-dependent components in fungal phytopathogens

At the molecular level, components of the ESCRT pathways are well understood in only a few model phytopathogenic fungi, including *F. graminearum* , *M. oryzae* and *Ustilago maydis* (Table 1). Of these, the two ascomycetes (*F. graminearum* and *M. oryzae*) are respectively responsible for Fusarium head blight of barley/wheat and rice blast disease (Xie et al., 2019a; Sun et al., 2022). The basidiomycetous species *U. maydis* is the causal agent of smut on maize and teosinte, a wild grass species considered the ancestor of modern maize (Bölker et al., 1992; Pérez-Rodríguez et al., 2021). Overall, these studies showed that the basic assembly and sequential recruitment of ESCRT components is likely consistent with that of the yeast. This is consistent with the fact that the ESCRT pathway is conserved with only some components apparently non-essential in certain lineages (Leung et al., 2008). Also, some ESCRT components are critical for growth, stress response reproduction system, while others affect pathogenesis.

Several gene knockout studies have been performed to determine the impact of ESCRT-associated genes in

these pathogens. A recent study by Xie et al., (2016) was the first to report on ESCRT-0 subunit Vps27 functions in *F. graminearum* . The authors revealed that Vps27 is crucial for the pathogen's development, conidiation and virulence. They also showed that ESCRT proteins -I, -II and -III are essential for endocytic delivery into the vacuole, therefore aiding in the production of mycotoxins (Xie et al., (2019b)). In *U. maydis* , the removal of ESCRT-III Did2 caused defects on hyphal growth as well as impaired conveyance of early and late endosomes (MVBs) (Haag et al., 2017). In*M. oryzae* , de-ubiquitination enzyme Doa4 is essential for ensuring pathogenicity and infection-related morphogenesis (Wang et al., 2018; Que et al., 2020).

The above studies on phytopathogenic fungi are consistent with those conducted on human pathogenic fungi. For example, in *C. neoformans,* Doa4 mutants exhibited reduced pathogenicity and phenotypic defects (Amerik et al., 2000a; Fang et al., 2012; Que et al., 2020). In*C. albicans* , deletion mutants of ESCRT-III SnF7 are associated with acute impairment on hyphal germination (Yang et al., 2020). Additionally, DUBs such as Ubp14, Ubp8 and Ubp4 have been shown to play critical roles in conidiation, growth and, most importantly, pathogenicity (Wang et al., 2018; Que et al., 2020 and Yang et al., 2020).

Table 1: Highlight of the critical ESCRT pathway components and their established roles in plant pathogenic fungal species Sub-complexes	Table 1: Highlight of the critical ESCRT pathway components and their established roles in plant pathogenic fungal species Yeast	Table 1: Highlight of the critical ESCRT pathway components and their established roles in plant pathogenic fungal species Plant pathogenic fungi	Table 1: Highlight of the critical ESCRT pathway components and their established roles in plant pathogenic fungal species Species	Table 1: Highlight of the critical ESCRT pathway components and their established roles in plant pathogenic fungal species Reported functions	Table 1: Highlight of the critical ESCRT pathway components and their established roles in plant pathogenic fungal species References
ESCRT-0	Vps27 Hse1	Vps27 Hse1	*Fusarium graminearum Magnaporthe oryzae*	Crucial for fungal development, conidiation and virulence and *[?]Fg vps27* displayed adverse growth defects and almost no aerial hyphae production Mutants of ESCRT-0 components disrupts growth, conidiation, pathogenicity and sexual reproduction and regulation of autophagy.	Xie et al., 2016 Sun et al., 2022

ESCRT-I	Vps23 Vps28 Vps37 Mvb12	Vps23 / Vps28	*Fusarium graminearum*	Loss of these genes led to severe growth defect therefore reduced virulence	Xie et al., 2019a
ESCRT-II	Vps36 Vps22 Vps25	Vps22, Vps25, Vps36	*Fusarium graminearum*	Aberrant endocytosis, decreased DON and reduced hydrophobicity.	Xie et al., 2019a
ESCRT-III	Vps20 SnF7 Vps24 Vps2	SnF7	*Magnaporthe oryzae*	Involved in pathogenic roles such as vesicle trafficking and endocytosis etc.	Cheng et al., 2018
		Did2 Vps60, Ist1 Ist1	*Ustilago maydis* *Fusarium graminearum* *Magnaporthe oryzae*	Defects in hyphal growth and impaired maturation and trafficking of early/late endosomes. Involved in vegetative growth, development, stress responses, virulence, endocytosis, and DON production. MoIst1 is involved in sporulation, appressorium development, plant penetration, pathogenicity, and autophagy in *M. oryzae*.	Haag et al., 2017 Xie et al., 2019b Sun et al., 2022
			Fusarium graminearum	Virulence, Endocytosis etc.	Xie et al., 2019b

End product of the ESCRT-pathway – EVs

In recent years, EVs have gained prominence in the field of biology due to their ubiquitous nature in organisms in all domains of life and their composition, thus making them appealing for better understanding

plant-microbe interactions (Kalluri and LeBleu, 2020). As a result, EVs have been characterized from cells across a spectrum of microbes including plant pathogenic fungi (e.g., *Penicillium digitatum*), bacteria (e.g., *Xanthomonas campestris pv., campestris)* and protozoans (e.g., *Trypanosoma cruzi*) (Chatterjee and Das, 1967; Raiborg et al., 2003; Sidhu et al., 2008; Torrecilhas et al., 2009; Coelho and Casadevall, 2019; Rybak and Robatzek, 2019; Costa et al., 2021). Presently, EVs are better characterized in human bacterial pathogens than any other microbes whereby they contribute to trafficking and delivery of effector proteins which induce host immune response (Rybak and Robatzek, 2019).

Studies on fungal EVs, especially those of plant pathogens, are gradually gaining momentum. Recently, a study by Costa et al., (2021) has demonstrated that EVs are used by filamentous fungal pathogens to export the phytotoxic compound, tryptoquialanines A, during plant infection and consequently cause tissue damage on citrus seeds. This is consistent with several other studies showing that EVs derived from fungal pathogens exert toxic effects on plant tissues (e.g., Silva et al., 2014; Bleackley et al., 2020). In other words, EVs may trigger a type of immune response in plants, which leads to hypersensitivity and subsequent discoloured areas on the plant leaf. This suggests the potential use of pathogen-derived EVs as plant improvement agents by serving as immune boosters or biological biomarkers.

EVs and plant-host interactions – the fungal pathogen viewpoint

The first report of EVs in a plant fungal pathogen came from the powdery mildew fungus *Blumeria graminis* (Hippe, 1985; Hippe-Sanwald et al., 1992). However, their earliest description was in 2007 from *C. neoformans* (Rodrigues et al., 2007; Samuel et al., 2015; Rodrigues et al., 2008a; Zhao et al., 2019). Nonetheless, the mechanism behind the production of EVs from the fungal cell wall is not well understood, although several mechanisms have been suggested (e.g., via passage channels, turgor pressure, cell-wall degrading enzymes, and viscoelasticity of the cell wall) (Wolf and Casadevall, 2014; Brown et al., 2015; Kuipers et al., 2018). For instance, a recent study on *S. cerevisiae* showed that EVs may contain enzymes and cell wall-related proteins believed to take part in cell wall remodelling, therefore, aiding their transition across the fungal cell wall (Zhao et al., 2019).

Whilst a shortfall of protocols and specific biomarkers for EV isolation continues to be a challenge in the field of EV biology, particularly for filamentous fungi, numerous studies on fungal EV production remain biased towards human fungal yeast pathogens (Albuquerque et al., 2008; Gehrmann et al., 2011; Vallejo et al., 2011; Vallejo et al., 2012; Vargas et al., 2015; Leone et al., 2017; Bielska et al., 2018; Ikeda et al., 2018; Peres da Silva et al., 2019; Lavrin et al., 2020). Consequently, very little is known about the release of EVs in plant pathogenic fungi, although a large number of studies have confirmed their presence in these organisms (Silva et al., 2014; Bitencourt et al., 2018; Liu et al., 2018; de Paula et al., 2019; Souza et al., 2019; Bleackley et al., 2020; Brauer et al., 2020; Rizzo et al., 2020; Costa et al., 2021; Garcia-Ceron et al., 2021). However, the production of EVs in fungi is considered unconventional because they mostly transport proteins lacking signal peptides (Samuel et al., 2015). For example, *B. graminis* secrete avirulence proteins (i.e., AVR_{a10} and AVR_{k1}) that are 'leaderless' (Samuel et al., 2015; Ridout et al., 2006). Mammalian studies have, however, reported the delivery of proteins with or without signal peptides by EVs, suggesting that this unconventional secretory pathway is a highly complex phenomenon (Samuel et al., 2015).

Fungal virulence is associated with the release of EVs that are involved in modulating acceptor cells. Thus, EVs are required for fungal pathogenesis due to their ability to modulate receipt cells to promote virulence (Rodrigues et al., 2007; Vargas et al., 2015; Joffe et al., 2016). In addition to their phytotoxic activity as mentioned previously, *F. oxysporum f. sp. vasifectum* EVs were found to comprise of virulent polyketide synthases and proteases etc. (Garcia-Ceron et al., 2021). Also, EVs in *C. neoformans* were found to contain virulence factors such as glucuronoxylomannan (GXM) and glucosylceramide (Rodrigues et al., 2007; Rodrigues et al., 2008a).

Several studies on plant pathogenic fungi including *M. oryzae* , and the oomycete pathogen *Phytophthora infestans* , revealed that the ESCRT pathway is utilized to export virulence-associated proteins via vesicles (Giraldo et al., 2013; Wang et al., 2017). The citrus fungal pathogen, *P. digitatum* , is reported to release

EVs of myriad cargos containing mycotoxins and alkaloids (tryptoquialanine A) during infection (Costa et al., 2021). Moreover, *B. cinerea* EVs carry sRNAs which promote pathogen infection by silencing the plant immunity genes, suggesting that EVs mediate the delivery of fungal virulent factors into host plants (Rodrigues et al., 2008b; Weiberg et al., 2013; Wang et al., 2016, 2018). EVs from fungal species such as*Histoplasma capsulatum* and *C. parapsilosis* , have also been shown to contain effector proteins, which may play a role in modulating their host immune responses during host-pathogen interaction (Albuquerque et al., 2008; Vargas et al., 2015; Gil-Bona et al., 2015). Taken together, these findings suggest that the role of EVs as carriers of virulence factors and immunomodulators is universal.

EVs and plant-fungal interactions – the plant host viewpoint

To survive, plants must respond quickly to its pathogens. As a result, certain intercellular changes, such as organelle rearrangement and cytoskeleton structural changes, may form a physical barrier that prevents infection at the site of attack. (Frey and Robatzek, 2009). Previously, plant EVs were reported to accumulate at the site of fungal infection and, thus, believed to be involved in trafficking molecules associated with defence between the plasma membrane and cell wall (Snetselaar and Mims, 1994; An et al., 2006; Samuel et al., 2015). Additionally, researchers have established that the secretion of plant EVs is enhanced during infection, which suggests involvement in some of the intercellular modifications (An et al., 2006; Wang et al., 2014 and Rutter and Innes, 2017). The cargo of plant EVs is sorted and packaged in response to the type of infection or injury, and it is significantly distinct to the cargo of fungal EVs secreted during infection (Samuel et al., 2015).

Knowledge on plant EVs is limited. Nonetheless, plant EVs have been reported to facilitate trafficking and delivery of large quantities of proteins lacking signal peptides to the extracellular environment (Regente et al., 2012; Pompa et al., 2017). Recent studies show that plants release EVs enriched with cell wall remodelling and defence related proteins in response to biotic or abiotic stress (Regente et al., 2009; Prado et al., 2014; Regente et al., 2017; Rutter and Innes, 2017; Baldrich et al., 2019). Rutter and Innes, (2018) also suggest that plant EVs presumably ferry RNAs into pathogens, thus demonstrating their potential as mediators of RNAs interspecies transmitters. Additionally, Baldrich et al., (2019) reported that EVs of *A. thaliana* convey diverse classes of sRNAs (i.e., microRNAs and small interfering RNAs) including a class of "tinyRNAs" (10-17 nt) whose role is not yet known but suspected to be associated with disintegrated artifacts of sRNA production. Koch et al., (2020) further demonstrate that EVs isolated from *A. thaliana* apoplastic fluids and leaf extracts enclosed transgene-derived sRNAs believed to be HIGs (Host induced silencing) related, which are transported during host-pathogen interactions. Defence genes targeted by HIGS have been reported to reduce *B. cinerea* virulence through silencing of its *Dicer-like 1* and*Dicer-like 2* genes (Wang et al., 2016).

EVs in fungal biofilm biology

Membrane vesicles play a significant role in the formation and maintenance of fungal biofilms (Zarnowski et al., 2018; Zarnowski et al., 2022). Biofilms are complex structures composed of communities of sessile cells embedded in a self-produced extracellular matrix (ECM) (Ramage et al., 2009; Harding et al., 2009). Formation of biofilms generally involves a succession of phases commencing with adherence of cells to surfaces, followed by the growth of cells which are covered by the ECM, leading up to maturation and dispersal of cells (Chandra et al., 2001; Harding et al., 2009). Nonetheless, the exact mechanisms by which membrane vesicles contribute to biofilm formation are not fully understood, but several mechanisms have been proposed. One proposed mechanism is that membrane vesicles are involved in the secretion of extracellular matrix (ECM) components, such as polysaccharides and proteins, which are necessary for the formation of the biofilm structure (Harding et al., 2009; Di Martino, 2018). Vesicles are also known to transport enzymes that are involved in the degradation of host tissues, which can create a suitable environment for biofilm formation (*Zarnowski*et al., 2018). Another proposed mechanism is that membrane vesicles are involved in intercellular communication within the biofilm. Studies have shown that vesicles can transfer signaling molecules, such as lipopeptides and small RNAs, between cells, which can coordinate the behavior of the cells within the biofilm (Leone et al., 2017). Additionally, membrane vesicles are also thought to play a role in the development of antifungal resistance. Vesicles have been found to sequester drugs and protect the biofilm cells from their

effects, and also to detoxify drugs, breaking them down before they reach the cells (et al., 2018; Zarnowski et al., 2022).

EVs are also involved in development of biofilms of some bacterial and yeast species (i.e., *Toxoplasma gondii etc.*) (Li et al., 2018, Zarnowski et al., 2022). Studies on the role of EV in biofilms in plant filamentous plant fungi have not been reported, although in yeasts (e.g., *C. albicans*) a number of studies have been conducted. Recent studies have shown that the structural complexity of biofilms (i.e., high cell density, ECM etc.) leads to production of EVs which can enhance drug resistance and are unique to planktonic (Free-living) cells (Bielska and May, 2019, Honorato et al., 2021, Zarnowski et al., 2022). The cargo of *C. albicans* biofilm EVs ferry compounds including cell-wall degrading enzymes as well as mannan and glucan (Zarnowski et al., 2014, Mitchell et al., 2015, Garcia-Ceron et al., 2021). Compatible with the assembly of the ECM, the delivery mannan-glucan complex by EVs is critical for drug resistance (Zarnowski et al., 2014, Mitchell et al., 2015). ESCRT-I genes including Hse1 and Vps27 were found enclosed in secreted EV cargo of *C. albicans* . These ESCRT-I containing EVs are reported to restore the biofilm matrix architecture and quantities of the key mannan–glucan components, an indication that they may function in biofilm EV production and promotion of matrix biogenesis (Zarnowski et al., 2018). EVs are also believed to control morphogenesis in *C. albicans* as their presence hinders the process of biofilm formation and dimorphic transition (Honorato et al., 2021). For instance, EVs containing RNA are assumed to actively take part in the dimorphic transition of *Pichia fermentans* (Leone et al., 2017). Overall, membrane vesicles are involved in multiple aspects of biofilm formation, including the secretion of matrix components, intercellular communication, and drug resistance.

Conclusions

Since the discovery of EVs, there has been a growing interest in the mechanisms involved in the sorting and packaging of their cargo (proteins, nucleic acids, and so on). The components of ESCRT pathway function together to deliver proteins into EVs. ESCRTs are thus crucial in plant pathogenic fungi as they contribute to their pathogenesis, developmental and growth phenotypes. We hypothesize that plant pathogens use the ESCRT pathway to sort and package protein lacking signal peptides, and that ubiquitin modification is necessary for fungal pathogenesis. The exact role of this pathway requires further studies as proteins with signal peptides are also reported in EVs. Few plant pathogen studies have highlighted the importance of ESCRT proteins, especially in the sorting of virulent factors into EVs. We suggest that the core functions of the ESCRT pathway genes need to be investigated further in plant pathogenic fungi, particularly since it has been demonstrated that ESCRT protein dysfunction causes aberrant endosomal compartments and reduced ILV formation. The much-needed push provided by genome sequencing makes it simple to characterize this system in fungi with completely sequenced genomes. Through the characterization and detection of particular cargos sorted by the ESCRTs, we can gain further insights into possible mechanisms involved in the pathogenesis of plant pathogen fungi. The ESCRT pathway is involved in the formation of MVBs, which release ILVs as EVs 'exosomes' loaded with bioactive molecules during heterotypic fusion. Intercellular contact, pathogenicity, and cellular homeostasis all depend on EVs. As a result, their release can serve as a barometer of disease, stress, or health. The information gained on the mechanisms involved in the pathogenicity effects of plant pathogenic EVs can serve as a stepping stone toward improving our ability to manage their damaging effects on agricultural crops and forests.

Credit authorship contribution statement

Francinah M Ratsoma: Conceptualization, methodology, investigation, analysis, writing-original draft, writing review and editing. Quentin C Santana: Methodology, investigation, analysis, writing review and editing. Brenda D Wingfield: Conceptualization, methodology, investigation, analysis, writing review and editing. Emma T Steenkamp: Conceptualization, methodology, investigation, analysis, writing review and editing Thabiso E Motaung: Conceptualization, Methodology, writing review and editing, resources, supervision and funding acquisition.

Funding details

The South African National Department of Science and Innovation-NRF under the Thuthuka funding in-

strument (Grant no. 129580) and the Centres of Excellence programme and South African Research Chairs Initiative (Grant No. 98353).

Conflict of interest

None

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