ER Membrane Contact Sites: Key Platforms for Biogenesis of RNA-Containing Extracellular Vesicles

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Abstract
The mechanisms by which cytoplasmic cargoes such as RNAs are incorporated into extracellular vesicles (EVs) are poorly understood. In a recent article published in Developmental Cell, we describe a novel function of endoplasmic reticulum membrane contact sites (ER MCS) in regulating biogenesis of RNA-containing EVs (Barman et al., 2022). We identified the ER MCS tether protein VAP-A and the ceramide transporter CERT as key drivers of this process. VAP-A depletion and overexpression produced corresponding changes in the overall number and RNA content of secreted EVs. Further sub-fractionation of small EVs from VAP-A depleted cells revealed a distinct loss in a specific subset of dense, RNA-loaded small EVs that are critical for the transfer of miR-100 to recipient cells. Cell imaging data confirmed the loss of RNA and RNA binding proteins (RBPs) in VAP-A-knockdown multivesicular bodies. Lipid analysis of VAP-A-knockdown EVs revealed decreases in ceramides, which are known to affect EV biogenesis. Depletion of the ceramide transfer protein CERT, which interacts with its binding partner VAP-A at ER MCS, leads to similar defects in EV number and RNA content as VAP-A-knockdown. These data suggest a model for ER MCS as platforms for biogenesis of a key EV population via ceramide transfer and RNA loading.

Keywords
endoplasmic reticulum, extracellular vesicles, exosomes, VAP-A, CERT, ceramide, RNA

Extracellular vesicles (EVs) are small membrane-enclosed transporters of protein, RNA, and lipid cargos that are released from cells to promote cellular communication. EVs are known to arise through two basic processes. Exosomes are generally 30–150 nm in diameter and are formed by inward budding of endosomes to form multivesicular bodies (MVBs). Ectosomes, also known as microvesicles, range in size from 100 nm to >1000 nm and are formed through outward budding of the plasma membrane (O’Brien et al., 2020). Due to the difficulty in identifying the originating membrane in EV preparations, purified EVs are often termed small and large EVs. With few exceptions, the molecular drivers and discrete steps in EV biogenesis are poorly understood; however, due to the biological and therapeutic impact, this is an area of intense investigation.

Numerous sequencing studies have revealed that EVs carry a diverse transcriptome, including mRNAs, microRNAs, and other small RNAs. RNA binding proteins (RBPs; e.g. Ago2, hnRNPA2B1, Syncrip, YBX1, ELAV, and La protein) have been established as key regulators, and specific microRNA motifs are required for some RNA sorting processes (O’Brien et al., 2020). However, it remains unclear what mechanisms govern how RNA-RBP complexes (ribonucleoprotein complexes, RNPs) are trafficked to and sorted into EVs.

To consider potential routes of RNP trafficking to EV biogenesis sites, we drew from our previous studies showing that microRNAs and Argonaute 2, a core component of the RNA-induced silencing complex, are physically associated with the cytoplasmic face of the endoplasmic reticulum (ER) before moving to MVBs (Barman and Bhattacharyya, 2015; Bose et al., 2017). Further connecting the dots, the RBPs hnRNPA2B1 and SYNCRIP that both play roles in

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Membrane contact sites (MCS) between the ER and other organelles act as key hubs for intracellular processes such as calcium and lipid exchange and organelle fission (Phillips and Voeltz, 2016). A number of tether proteins mediate these contacts, controlling molecular signalling and exchange through protein-protein interactions that bridge the contact sites (Phillips and Voeltz, 2016). One of these tether proteins, vesicle associated-membrane-protein-associated protein A (VAP-A), binds endosome-localized lipid transport proteins and functions in lipid transfer from the ER to endosomes and other organelles, including the plasma membrane (Phillips and Voeltz, 2016). Based on these pieces of data, we hypothesized that ER-associated RNPs are incorporated into newly forming EVs at ER-endosome and/or ER-plasma membrane MCS. To test the link between ER MCS and biogenesis of RNA-containing EVs, we depleted VAP-A by shRNA from colon cancer cells and confirmed disruption of MCS formation by both transmission electron microscopy and proximity ligation (Barman et al., 2022). The VAP-A knockdown cells exhibited significant declines in the number, size, and RNA content of secreted EVs without detectable effects on cell viability or ER stress. Furthermore, RNA-seq data revealed a loss of specific RNA cargoes in EVs purified from VAP-A knockdown cells. As the EVs released from control and VAP-A knockdown cells are a heterogeneous mixture of EVs differing in character and cargo, we sub-fractionated the EVs using density gradient centrifugation and identified a dense small EV population enriched with RNAs that is specifically reduced following VAP-A knockdown. We also exploited a GFP-tagged constitutively active form of Rab5 (Rab5 Q79L) that induces formation of enlarged endosomes to monitor intraluminal filling of MVBs. This approach demonstrated that VAP-A knockdown leads to decreased loading of the microRNAs let-7a and miR-100, the RBPs Ago2 and SYNCRIP, and the EV marker CD63 into MVBs. Although Rab5 Q79L-labeled structures are not an exact counterpart of unperturbed MVBs, our findings are bolstered by previous studies that corroborate the colocalization of CERT, RBPs, and miRNAs with CD63-positive MVBs (Fukushima et al., 2018; O’Brien et al., 2020).

To test whether the pronounced effects of VAP-A on EV biogenesis have functional consequences, we employed two experimental models: miRNA transfer assays and a
xenograft tumor model treated with purified EVs from control or VAP-A knockdown cells. Using luciferase assays of miR-100 transfer, we verified that the subpopulation of EVs controlled by VAP-A is critical for functional delivery of miR-100 to recipient cells. We also observed defects in the growth of xenograft tumors derived from VAP-A knockdown cells, which could be rescued by co-implantation of control but not knockdown small EVs. While there is much more to be done, these assays indicate that the subpopulation of EVs formed at ER MCS is functionally active.

As VAP-A binds various lipid transporters, we hypothesized that lipid transport between membranes might be involved in the EV biogenesis process. Notably, ceramide formation can induce membrane curvature and EV biogenesis (Trajkovic et al., 2008), and Ceramide transporter protein (CERT) is a VAP-A-binding partner that transfers ceramide from the ER to the Golgi (Hanada et al., 2003; Phillips and Voeltz, 2016). Recently, CERT was also shown to mediate ceramide transport at ER-endosome contact sites that mediate EV biogenesis (Trajkovic et al., 2008), and Ceramide transporter protein (CERT) is a VAP-A-binding partner that transfers ceramide from the ER to the Golgi (Hanada et al., 2003; Phillips and Voeltz, 2016). Recently, CERT was also shown to mediate ceramide transport at ER-endosome MCS, altering EV secretion from palmitate-stimulated hepatocytes (Fukushima et al., 2018). Indeed, lipidomics mass spectrometry data uncovered distinct classes of ceramides that were reduced in the EVs we purified from VAP-A depleted cells (Barman et al., 2022). In addition, CERT depletion virtually phenocopied the effects of VAP-A depletion on EV formation and RNA content. While ceramides are synthesized in the ER, they can also be formed through sphingomyelin hydrolysis by neutral sphingomyelinase 2 (nSMase2), promoting EV biogenesis (Trajkovic et al., 2008). High resolution confocal z-stacks of cells expressing GFP-VAP-A and mCherry-Rab5 Q79L and immunostained for nSMase2 revealed that VAP-A positive ER was positioned as a bridge between MVBs and nSMase2-positive ER-associated structures. Together, these findings identify VAP-A-CERT linkages at ER MCS as key biogenesis regulators of RNA-containing EVs. Our study joins several others showing that ER MCS are key locations for biogenesis of diverse kinds of EVs (Figure 1). In addition to ceramide transport (Barman et al., 2022; Fukushima et al., 2018), VAP-A linkages to the endosomal cholesterol transporter ORP1L also affect EV biogenesis and cargo content, regulating formation of EVs containing extracellular matrix (Albacete-Albacete et al., 2020) and epidermal growth factor receptors. (Eden et al., 2016). Further studies employing super resolution or correlative light-electron microscopy are necessary to visualize the tripartite event where ER, endosomes, and RNA-RBP complexes converge to induce incorporation of RNA into newly forming intraluminal vesicles. The timing and frequency of these events are also yet to be determined as such measurements would require fast, super resolution live cell imaging. Finally, as many RNA granules are associated with the ER and carry out diverse functions, future studies should also address the source(s) of the RNA and the cellular context that controls RNA incorporation into EVs.

Declaration of Conflicting Interests
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